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BIOTECHNOLOGIA ACTA

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Vol. 6, No 4, 2013

Special issue

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ЗМІСТ

СОДЕРЖАНИЕ

From the Editor-in-chief

Dear friends and colleagues, those who read these lines in *Biotechnologia Acta!*

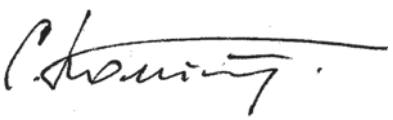
Please trust me that I am writing them with special feelings. 70 years is a wonderful opportunity to stop, at least for a moment, in the middle of every day «whatever activities» and to recall in the memory the most important and most pleasant events that had happened during the life. And meetings with colleagues members of the scientific community are indeed among the most enjoyable of these memories.

This year we had a set of meetings where I met many friends. In particular, it was during FEBS Congress-2013 in Saint Petersburg, at Parnas Conference in Jerusalem, in October 2013 and, certainly, during our «Jubilee» Conference «Biochemistry and Biotechnology for Modern Medicine» in Kyiv, on September 19–20. While organizing the Kyiv Conference, the Conference Organizing Committee in Kyiv has collected articles from possible lecturers (participants of the Conference) and decided to publish them in a book under the Conference title. Because the articles published-in the book might not be widely available for the scientific community, it was also decided to divide these articles in two sets and to publish them in Palladin Institute's two scientific periodicals, *Biotechnologia Acta* and *Ukrainian Biochemical Journal* depending on the content. And here is the result — you hold the Journal

I know personally well all the authors (or principal authors, if they are several per article). With some of them, we are friends for over 40 years, with others not for so long, but in any case we all are united by the wonderful feeling of a friendly scientific community, by respect to each other and by our common cause to which we have devoted our lives s to Biomedical sciences, though among contributors one may find biologists, chemists, and medical doctors. So, I take this opportunity to express my most sincere thanks to my friends scientists from abroad (working in 10 countries!), to my Ukrainian colleagues and to the coworkers from our Institute of Biochemistry who were so kind to find time and opportunity and write articles in my honour. Neither the Conference nor the book or journal issues could happen without everybody's good will.

Most of the articles in this issue are devoted to scientific themes on which I was either working myself with my coworkers or which were in the scope of my scientific interests. Besides, it is pleasant to note that there are very young but talented scientists among the authors being mostly very famous scientists and members of academies of sciences all over the world. I am confident that they will become well known to the world scientific community pretty soon, and I may only wish them (and myself as well) to follow my good friend former President of the Weizmann Institute, professor Michael Sela as an example of dedication to Science and of active scientific longevity. He took part in FEBS Congress-2013, in our Conference «Biochemistry and Biotechnology for Modern Medicine» in Kyiv on September 19–20, in Parnas Conference in Jerusalem. His article has been published both, in the book and in this issue of *Biotechnologia Acta*.

And the last but not at all the least. My special and deepest gratitude to the team from Palladin Institute of Biochemistry, Dr. Valentina Danilova, head of the team, Mrs. Alyona Vinogradova, and Dr. Evgeniy Levitzky (*Biotechnologia Acta*) who did all the job for this journal to become a reality. Thanks to all of you!



S. Komisarenko

Від Головного редактора

Шановні друзі та колеги, хто читає ці рядки в «Biotechnologia Acta»!

Повірте, я пишу їх з особливими почуттями. 70 років — це чудова нагода зупинитися, хоч не надовго, у вирі повсякденної діяльності й переглянути у пам'яті найбільш важливі та приємні події, що трапились у твоєму житті. І саме зустрічі з колегами по науковому товариству є чи не найприємнішими серед цих спогадів.

Цього року було декілька нагод зустрітися з багатьма друзями-вченими. Зокрема, в липні у Санкт-Петербурзі під час Конгресу FEBS-2013, на Парнасівській конференції в Єрусалимі (у жовтні) та, звичайно, на нашій «ювілейній» конференції «Біохімія і біотехнологія — сучасній медицині», 19–20 вересня у Києві. У період підготовки конференції в Києві Організаційний комітет зібрав статті можливих лекторів-учасників і вирішив опублікувати їх у книзі під назвою цієї конференції. З огляду на те, що видана в Україні книга може бути малодоступною для наукового загалу, було вирішено розділити статті за змістом на дві частини і надрукувати їх відповідно у двох наукових часописах Інституту біохімії ім. О. В. Палладіна — у «Biotechnologia Acta» та в «Українському біохімічному журналі». А ось і результат — Ви тримаєте в руках журнал.

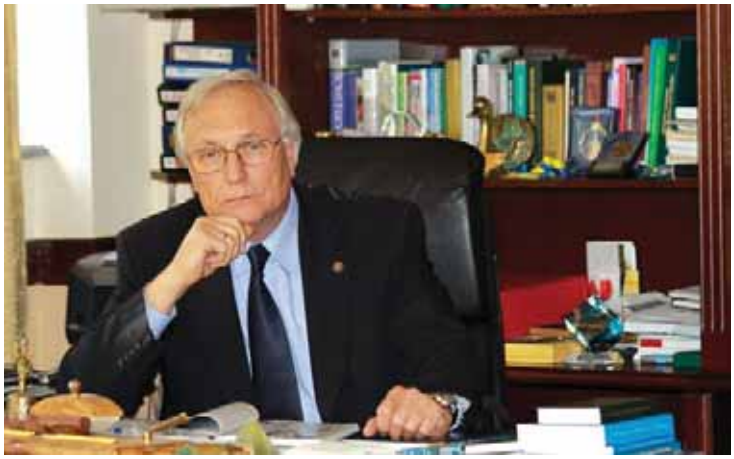
Я особисто добре знайомий з усіма авторами чи співавторами (якщо їх декілька у статті). З деякими із них товаришую понад 40 років, із деякими — не так довго, однак усіх нас об'єднує чудове почуття дружньої колегіальності, повага один до одного й те, що ми присвятили своє життя спільній справі — медико-біологічним наукам, хоча в авторському колективі є і біологи, й хіміки, і медики. Тож я користуюсь нагодою, аби висловити щиру подяку — моїм друзям-ученим з-за кордону (які працюють у 10 країнах світу!), моїм українським колегам та співробітникам нашого Інституту, які були настільки ласкаві, що знайшли час і можливість написати статті на мою честь, і за це я їм щиро вдячний. Без доброї волі кожного з них не було б ані цього журналу, ані книги.

Переважну більшість статей моїх колег присвячено науковим темам, якими я безпосередньо займався зі своїми співробітниками в Інституті біохімії чи які були у колі моїх наукових інтересів. Приємно також відзначити, що серед авторів, де переважають відомі вчені, більшість яких — члени академій наук різних країн, є зовсім молоді, але талановиті вчені, які, на моє переконання, невдовзі стануть відомими науковому світові. І тут я їм (а також і собі) можу тільки побажати брати приклад з мого давнього друга — колишнього Президента Вайцманівського інституту, професора Майкла Села, який є зразком відданості Науці та активного наукового довголіття. Він брав участь і в Конгресі FEBS-2013, і є учасником нашої конференції в Києві, і Парнасівської — в Єрусалимі, а його статтю надруковано і в книзі, і в цьому номері «Biotechnologia Acta».

І нарешті останнє, але вкрай важливе. Моя особлива та щира подяка «команді» Інституту біохімії ім. О. В. Палладіна НАН України — лідеру «команди» к. б. н. В. М. Даниловій, а також А. С. Виноградовій, Г. М. Шевченко і д. б. н. Є. Л. Левицькому («Biotechnologia Acta»), які доклали чимало зусиль аби цей часопис став реальністю. Дякую їм усім!

С. Комісаренко

СЕРГІЮ ВАСИЛЬОВИЧУ КОМІСАРЕНКУ — 70!



9 липня 2013 р. виповнилося 70 років від дня народження видатного українського вченого в галузі біохімії та молекулярної імунології **Сергія Васильовича Комісаренка** — академіка Національної академії наук України та академіка Національної академії медичних наук України, академіка-секретаря Відділення біохімії, фізіології і молекулярної біології НАН України, директора Інституту біохімії ім. О. В. Палладіна НАН України (1989–1992 та з 1998 дотепер), головного редактора наукового журналу «Biotechnologia Acta».

Народився С.В. Комісаренко в м. Уфа (Російська Федерація) в сім'ї відомого українського вченого-патофізіолога, згодом академіка, засновника і першого директора Інституту ендокринології та обміну речовин АН УРСР Василя Павловича Комісаренка, ім'ям якого названо цей Інститут. Мати — кандидат економічних наук, була дуже доброю, вірною і люблячою дружиною, зуміла створити в родині атмосферу, сприятливу для роботи свого чоловіка, з яким прожила разом у любові та злагоді понад 60 років. Батьки приділяли багато часу вихованню дітей, з раннього дитинства прищеплювали їм інтерес до знань та культурної спадщини, вчили, що потрібно багато й наполегливо трудитися, не нав'язували своїх поглядів, а пояснювали, що обирати життєвий шлях доведеться їм самим, і шлях цей довгий, на якому вони зустрінуть не тільки радощі, а й перешкоди, певні труднощі, які потрібно буде здолати. Частими гостями в їхній домівці були видатні особистості, вчені, відомі діячі науки і культури. Високоінтелектуальні бесіди поважних людей стосовно досягнень у різноманітних галузях науки і техніки, культури, політики, мистецтва безумовно вплинули на формування світогляду і подальший життєвий та професійний вибір дітей.

Старший брат став лікарем. Нині він — доктор медичних наук, член-кореспондент Національної академії медичних наук України, двічі лауреат Державної премії УРСР, лідер хірургічної ендокринології у країні.

А молодший, Сергій Васильович, якого, під впливом, за його словами, атмосфери «високої» медицини, що панувала в батьківському домі, цікавили механізми життєдіяльності живих істот, прислухався до поради батька і після закінчення школи вступив на лікувальний факультет Київського медичного інституту (нині Національний медичний університет імені О. О. Богомольця).

На третьому курсі, аби опанувати практичну медицину, майбутній учений протягом року працював ночами на Київській міській станції швидкої допомоги фельдшером. 1966 року закінчив із відзнакою Київський медичний інститут, 1969 року — аспірантуру Інституту біохімії АН УРСР, з яким пов'язано усе творче життя ювіляра. Саме тут він пройшов усі щаблі наукової кар'єри — від аспіранта, молодшого і старшого наукового співробітника, вченого секретаря, завідувача лабораторії, завідувача відділу до директора цього Інституту.

Уже з молодих років Сергій Васильович намагався одержати якомога більше знань у різних галузях науки. Одночасно з аспірантурою навчався на механіко-математичному факультеті Київського державного університету ім. Т. Г. Шевченка й поглиблював свої знання з англійської і французької мов, ніби передчуваючи, що в подальшому житті вони стануть йому у великій пригоді.

Для істинного науковця обов'язковою умовою наукового зростання є тривале стажування у провідних науково-дослідницьких центрах світу. Окрім численних короткострокових закордонних наукових відряджень, С.В. Комісаренко працював в Інституті Пастера у Парижі (1974–1975 рр.) та в Нью-Йоркському протиракковому центрі ім. Слоан Кеттерінг (1981 р.). Йому належить ідея встановлення від імені Академії наук УРСР пам'ятника Іллі Мечникову в Інституті Пастера в Парижі (1986 р.).

Наукові інтереси Сергія Васильовича охоплюють різні галузі науки. Це — біотехнологія та біохімія, молекулярна імунологія і фармакологія, медична та біоорганічна хімія, фізіологія і молекулярна біологія тощо.

С.В. Комісаренка заслужено вважають засновником молекулярної імунології в Україні. Головні напрями його наукової діяльності пов'язані з імунохімічним дослідженням антигенної структури протеїнів та пептидів. Першим у колишньому СРСР він розпочав вивчення імунохімічної структури пептидів і протеїнів, упровадив методи імуноензимології і проточної цитофлуориметрії та одним із перших увів у дослідження гібридомну техніку одержання моноклональних антитіл. Велику увагу приділяв вивченню біологічної дії фосфорорганічних комплексонів — бісфосфонатів. З використанням фосфонатів, цитотоксичних антибіотиків та антитіл (або їхніх фрагментів) проти мембранних антигенів було створено імуновекторні молекули для вибіркового лізису пухлинних клітин, встановлено антигенну структуру нейротоксину апаміну, цитохрому *c* та деяких інших протеїнів і пептидів, зокрема протеїнів системи зсідання крові, антигенів мікобактерій, збудників кашлюка й дифтерії. С.В. Комісаренко є засновником лабораторії імунохімії (1975), яку 1982 року перетворено на відділ молекулярної імунології Інституту біохімії АН УРСР. За результатами здійснених у відділі під керівництвом С.В. Комісаренка фундаментальних досліджень було створено сучасні діагностичні методи для моніторингу стану системи зсідання крові та оцінки небезпеки тромбоутворення, а також діагностичні набори для виявлення туберкульозу і дифтерії, визначено протипухлинну та імуномодулювальну активність метиленбісфосфонової кислоти, на основі якої створено препарат «Мебіфон» для лікування пухлин передміхурової та молочної залоз, який успішно пройшов клінічні випробування і впроваджений у практику онкологічних клінік України (випускає фармоб'єднання «ФАРМАК»). Запропоновано й терапевтичні чинники для запобігання тромбоутворенню. За ініціативою С.В. Комісаренка розроблена і вже готова до впровадження технологія отримання високоочищених і вірусобезпечних антигемофілійних препаратів із крові людини, створено унікальний препарат «Мебівід» для лікування остеопорозу. Також тут уперше в Україні було створено бібліотеку рекомбінантних одноланцюгових антитіл людини (потужністю 10 млрд. антигенних специфічностей).

Важливим етапом у житті С.В. Комісаренка були дослідження, пов'язані з аналізом наслідків аварії на Чорнобильській АЕС. Під його керівництвом проведено унікальне обстеження людей, які працювали там після цієї катастрофи, і вже наприкінці 1986 року вперше було встановлено і доведено (всупереч офіційній думці, яка існувала в ті роки), що низькі дози радіації суттєво пригнічують систему природного імунітету, зокрема знижують кількість та активність природних клітин-кілерів, які відповідають за протипухлинний та противірусний імунітет у людини. Є підстави вважати, що зростання кількості онкологічних захворювань після аварії на Чорнобильській АЕС зумовлено не лише безпосереднім мутагенним впливом випромінювання, але й значною мірою саме імуносупресивною дією іонізуючого випромінювання. У цьому зв'язку С.В. Комісаренко вперше застосував термін «Чорнобильський СНІД». За роботи з екології, присвячені дослідженню Чорнобильської катастрофи, Рада Кінгстонського університету у Великій Британії обрала 1997 року С.В. Комісаренка почесним доктором свого університету.

Водночас із науковою роботою Сергій Васильович проводить також науково-організаційну та педагогічну діяльність, приділяючи велику увагу професійній підготовці молодих науковців. У 1978–1986 рр. він успішно керував республіканською міжвідомчою науковою програмою з імунології «Механізми імуностимуляції», був організатором республіканських шкіл з молекулярної імунології, упродовж багатьох років читав курс лекцій з імунохімії в Київському державному університеті імені Тараса Шевченка та з молекулярної імунології — у Київському від-

діленні МФТІ. Зараз очолює Відділення «Біотехнологія» кафедри біохімії Київського національного університету імені Тараса Шевченка.

Залишається лише дивуватися вмінню С.В. Комісаренка поєднувати професійну наукову роботу з державною і громадською діяльністю. 1990 року Верховна Рада УРСР обрала його заступником Голови Ради Міністрів УРСР. За час перебування на посаді заступника Прем'єр-міністра України з гуманітарних питань (до квітня 1992 року) С.В. Комісаренко брав активну участь у розробленні перших законів України з гуманітарних питань, зокрема про освіту, національні меншини, свободу совісті, пресу і засоби масової інформації, соціальне забезпечення тощо. Глибоко розуміючи серйозність ситуації з поширення ВІЛ/СНІДу в Україні, ініціював підготовку та прийняття Закону України про боротьбу зі СНІДом, створив та очолив урядову комісію боротьби зі СНІДом, організував Державний комітет боротьби із цією патологією. Сергій Васильович був головою низки урядових комітетів і комісій, зокрема з відзначення трагедії у Бабиному Яру (1990–1991 рр.), із проведення Конгресу українців (1991–1992 рр.), з гуманітарної допомоги (1992 р.), урядової протиепідемічної комісії та ін.

Важливою віхою в діяльності С.В. Комісаренка була його робота на посаді першого Посла України у Сполученому Королівстві Великобританії та Північної Ірландії (із травня 1992 до квітня 1998 року). С.В. Комісаренко не лише максимально сприяв розвиткові двосторонніх українсько-британських стосунків, але й ініціював вступ України до директорату Європейського банку реконструкції та розвитку (1993 р.), до Міжнародної морської організації (1995 р.) та інших важливих міжнародних організацій, розташованих у Британії, заснував у Лондоні благодійний фонд допомоги постраждалим від аварії на Чорнобильській АЕС (1993 р.), а також Британо-Українську торговельну палату (1997 р.). Посол С.В. Комісаренко успішно лобіював підтримку Британською делегацією прийняття України до Ради Європи, ратифікацію Британським парламентом Угоди про співробітництво між Україною та Європейським Союзом. Завдяки його активній діяльності як Посла Україна одержала у власність чотири будинки (три у Лондоні і один — в Единбурзі), чим було заощаджено декілька мільйонів фунтів стерлінгів. Назавжди залишиться у пам'яті громадян України той факт, що він домігся безкоштовної передачі Україні у 1995 році Британської Антарктичної станції «Фарадей» (зараз станція «Академік Вернадський»), відвідати яку Сергію Васильовичу пощастило тільки через 15 років. Діяльність Посла України у Британії високо оцінено в обох країнах. 1996 року С.В. Комісаренка було нагороджено відзнакою Президента України «За заслуги» III ступеня та номіновано кращим дипломатом року. У Британії у 1997 році його було обрано Почесним доктором двох університетів: за вагомі, саме наукові, досягнення — Почесним доктором Кінгстонського університету, а за дипломатичну діяльність — Почесним доктором Північно-Лондонського університету.

Після завершення дипломатичної місії та повернення до Києва у квітні 1998 року Сергія Васильовича було знову обрано директором Інституту біохімії ім. О. В. Палладіна НАН України, а у квітні 2004 року — членом Президії Національної академії наук України та академік-секретарем Відділення біохімії, фізіології і молекулярної біології НАН України. Широким є спектр його громадської діяльності: перший заступник Голови Української ради миру (1999), Президент Українського біохімічного товариства (1999), Президент Українського Інституту миру і демократії (2000), член Ради міжнародного журналу «Європа» (Польща), Голова Наглядової ради Міжнародного фонду Національної пам'яті України (2007), Почесний член та член Ради директорів Британо-Української торговельної палати, Президент благодійної організації інвалідів «Спеціальна Олімпіада України» (2002). Пропозиція очолити Спеціальну Олімпіаду України виявилася повною несподіванкою для академіка. Цей рух було засновано родиною Президента США Дж. Ф. Кеннеді з метою підтримки і соціальної адаптації людей з проблемами розумового розвитку. І знову Сергій Васильович проявив себе як чуйна, порядна людина, адже прийти на допомогу кожному, хто потребує його підтримки та доброго слова, то є для нього справою честі.

Останнім часом учений здійснює наукове керівництво дослідженнями, пов'язаними з проблемами біологічних загроз для людства і, зокрема, обґрунтуванням шляхів зменшення їх в Україні. Він організував низку міжнародних конференцій і семінарів із проблем біобезпеки і біозахисту, а з 2005 року очолює делегацію України на зустрічах експертів держав-учасниць Конвенції із заборони біологічної і токсичної зброї. Указами Президента України у вересні 2007 року і червні 2009 року С.В. Комісаренка призначено Головою Комісії з біобезпеки та біологічного захисту при Раді Національної безпеки і оборони України. Зараз С.В. Комісаренко фактично є головним експертом із питань біобезпеки в Україні.

Сергій Васильович передбачив важливу роль Парнасівських конференцій з проблем біохімії як шляху до встановлення широкомасштабних контактів українських учених-біохіміків з ученими Європи, а далі — з ученими США та інших високорозвинених країн світу.

С.В. Комісаренко започаткував видання в Україні нового наукового журналу «Біотехнологія» (зараз «Biotechnologia Acta»), який сприяє активізації розвитку цього розділу біологічної науки в нашій країні. Він є також головним редактором «Українського біохімічного журналу», членом редколегій міжнародного журналу «Європа» (Польща) та журналу з імунофармакології (Італія), членом Ради Міжнародного союзу біохіміків і молекулярних біологів (IUBMB), Федерації європейських біохімічних товариств (FEBS) і Міжнародного товариства імунофармакологів (США).

Важко перелічити всі титули і відзнаки С.В. Комісаренка. Він — академік Національної академії наук України та академік Національної академії медичних наук України, а також член Президії цих академій, лауреат Державної премії України (1979 р.) і премій НАН України імені О. В. Палладіна (2003 р.) та імені І. І. Мечникова (2011 р.). Удостоєний Почесних відзнак Президента України: орденів «За заслуги» III ступеня (1996 р.), II ступеня (1998 р.) і I ступеня (2013 р.), ордена князя Ярослава Мудрого V ступеня (2005 р.), Почесної Грамоти Верховної Ради України (2003 р.), а також ордена «Дружба» Китайської Народної Республіки (2012 р.); має почесне звання «Заслужений діяч науки і техніки України» (2008 р.).

С.В. Комісаренко — почесний член Польського біохімічного товариства (2011 р.). Має дипломатичний ранг Надзвичайного і Повноважного Посла України (1992 р.).

З нагоди ювілею ми, співробітники Інституту біохімії ім. О. В. Палладіна НАН України, з радістю відзначаємо, що маємо щастя працювати з такою Людиною, яка випромінює доброту, невичерпний ентузіазм, творчу активність, яскраву наукову ерудицію, виявляє наполегливість у досягненні поставленої мети і, водночас, є глибоко порядним, скромним, уважним і толерантним до наукового та іншого інакомислення. До цього слід додати іскристий гумор, такт, уміння вислухати й зрозуміти проблеми кожного працівника.

Можна тільки дивуватися, як за такого щільного графіка роботи Сергію Васильовичу вдається віднаходити час, що залишається від наукової діяльностіч, адміністративних та громадських обов'язків, для сім'ї, занять спортом, насолоджуватися звуками музики, милуватися живописом, спілкуватися з друзями...

Вельмишановний і дорогий наш Сергію Васильовичу!

Редакційна рада, редакційна колегія та редакція журналу «Biotechnologia Acta» щиро вітають Вас, дорога наша Людино, з ювілеєм. Бажаємо міцного здоров'я, невичерпної енергії, довгих, плідних років життя, сповнених добрими справами і вагомими творчими здобутками, наснаги, радості від звершеного, талановитих учнів і нових успіхів в ім'я розвитку вітчизняної науки на благо усього людства!

Бажаємо жити у силі й здоров'ї ще многії літа!

Щасливої долі!

"A MAN OF FOUR" — MR. KOMISARENKO IN MY EYES

HUANMING YANG
BGI-China

It is difficult for a man to be both a good professor and a good diplomat.

It is very difficult for a man to be both a good researcher and a good politician.

It is very very difficult to be a man of all the four, a good professor and a good researcher, a good politician and a good diplomat.

Even it is very rare, I have found such one, a man of the four, the only man I have ever met in my life until now in this world, Mr. Serhiy Komisarenko, in Ukraine.

I have just met Serhiy a few times, in Beijing and in Seville, in my institute and at an international meeting. I quickly recognized him comprehensive and could not but admit his great. I was not only impressed by his professional knowledge and visions, but also by his professional manner or style, a style of a general professor or a researcher.

It is well known that he has been one of the most versatile researchers in the field of immunochemistry at the international level, and has contributed to the field extensively. Also, it is all known to us that he is prolific researcher and has multiple research directions, mainly in immunochemical analysis of proteins and peptides, in molecular mechanisms of lymphocyte activation and in interrelation between protein immunochemical structure and their biological function. His contribution to biosafety is widely acknowledged that, under his guidance, it was found for the first time that low doses of radiation depressing the natural immunity in human, when Chornobyl Nuclear Plant exploded in 1986. His books, his hundreds of scientific papers in international journals and hundreds of lectures at international meetings, as well as his numerous awards including the most prestigious one for non-Chinese by the Chinese Central Government, have enhanced his global reputation in the international scientific community as somebody like me (even far worse), so called a researcher, or a scientist.

It is also all known that he has been teaching for decades in Kyiv State University and many other universities, on many subjects in the wide fields of life science and biotechnology. His students, of bachelor, master, and Ph. D.s, now «at every corner under the heaven» in Ukraine and many other countries, would all acknowledge that he has shaped, at least helped shape, their life and careers as somebody like me, a professor or a «teacher» as it is called in Chinese.

It is usual for a researcher and/or a scientist to hold some administrative positions, like Serhiy's numerous titles as secretary, head, or director of a department, institute, or a university. It is also not that unusual for some of them to be at high leveler, as a secretary of an academy who is responsible for many divisions or Institutes, like some of my friends. However, it was a really nice surprise to me when I was told that he is one of the most important statesmen in Ukraine. He is the former Deputy Chairman of Council of Ministers, Deputy Prime Minister responsible for humanitarian sector including health care, culture, education, science, social security, etc. almost everything I know and am interested in. He was even elected as a candidate to the post of the President of Ukraine. I can promise now that I had had voted for him if I had been a voter.

It is the general impression of scientists to the public that they are always thinking, or pretending to be thinking, in other words, not so social. It is known that not every politician is a qualified diplomat. Again, Serhiy is both. I was more surprised that he is a professional diplomat, the former Ambassador of Ukraine to several important countries such as UK and Ireland.

If you are also one of his friends, after listening to his stories about his deeply loved nation, after reading his articles on Ukrainian culture and politics, you would understand why he is the right person to be born in the right country and live in the right time in its history, and has chosen the right professions to have made the right contribution to his great nation, as well as to the whole world, and why only he can be such "a man of four".

It is indeed a privilege to be a friend and a colleague of Mr. Komisarenko, a real «man of four».

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ON THE NOTION OF SYNERGY OF MONOCLONAL ANTIBODIES AS DRUGS

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History of developing synergy between monoclonal antibodies, anti-tumor activity of monoclonal antibodies against tyrosine-kinases receptors EGFR/ErbB-1 and HER2/ErbB-2 as well as growth factor VEGF in various combinations are considered in the article. There were proposed hypotheses about potential molecular mechanisms underlay synergy between monoclonal antibodies (for homo- and hetero combinations of antibodies appropriately specific for antigenic determinants on the same or different receptors). Future trends in researches necessary to deeper understanding causes of this phenomenon and perspectives for practical application of monoclonal antibodies acted synergistically as immunotherapeutic drugs for human tumors treatment are reviewed.

Key words: monoclonal antibodies, epidermal growth factor receptors EGFR/ErbB-1 and HER2/ErbB-2, vascular endothelial growth factor (VEGF), synergy, molecular mechanisms, tumor immunotherapy.

Efforts to initiate a specific approach to immunotherapy of cancer started with the attachment of chemotherapeutic drugs by a weak covalent link to antitumor antibodies, at that stage still polyclonal [1–3]. Spacers such as dextran or polyglutamic acid were used to allow high drug load. At a later stage the antibodies were biotinylated, whereas the drug was attached to avidin, allowing a two stage drug targeting to the tumor [4]. During these experiments we noted that there was no need to covalently attach the drug to the antibody, which by itself had some antitumor activity. For example, when monoclonal antibodies (mAbs) to epidermal growth factor receptor (EGFR) were injected together with cisplatin [5] they exerted a strong synergistic effect on the ability to reduce the size of tumors (KB human epidermal carcinoma). This early observation, of a synergistic effect on cancer between an antibody and a chemotherapeutic drug, has paved the way for an extensively used clinical protocol [6] .

To further explore ways to enhance therapeutic efficacy, we addressed the mechanism underlying tumor inhibition by mAbs to receptor tyrosine kinases such as EGFR/ErbB-1 and HER2/ErbB-2. One mechanism attributes tumor growth inhibition to the ability of anti-receptor mAbs to induce endocytosis and degradation of the receptors. The mAbs down-regulate the receptor leading to attenuated ligand-induced signaling potency and duration. To enhance antibody-mediated endocytosis of these cancer-causing receptors we introduced combinations of mAbs and found that epitope-distinct mAbs to the same receptor (homo-combination) can significantly enhance the rate of receptor breakdown in KB cells over-expressing EGFR [7]. Further, when combined, the mAbs synergize in terms of growth inhibition of N87 human gastric carcinoma over-expressing HER2 [8]. The combinations act in synergy if they are directed against distinct epitopes, i.e. sufficiently remote from each other on the receptor. The mAbs then cross-link the receptors and efficacy of immunotherapy is attributed to receptor cross-linking and size of antibody-receptor clusters formed at the cell surface. The clusters are rapidly removed, a step which dictates the rate of endocytic clearance, receptor down-regulation and extent of signaling blockade [6].

A mechanistically distinct approach simultaneously targets two different receptors, such as targeting both EGFR and HER2 (hetero-combination) or a receptor (e.g., HER2) and an anti-angiogenic growth factor (VEGF,

using Avastin-bevacizumab). The extracellular domain of human ErbB presents adjacent or over-lapping determinants harboring multiple antigenic sites. Depending on the site, a mAb can be dormant, propagate tumor growth or mediate a distinct detrimental effect. It can disturb ligand binding, interfere with heterodimer formation that induce signal transduction, or interfere with any other pathway not yet identified. Scientific rationale suggests that combining two mAbs to two epitopes on the same receptor, or two mAbs to the two receptors, can target different pathways. They may perturb the cancer cell by inducing a collaborative damage of simultaneously impaired functions often by differing but complementary mechanisms of action of the two mAbs.

While conducting experiments with various combinations of antibodies to HER2, we noted an interesting observation: an antibody which by itself exerted no effect on tumor growth in animals was nevertheless able to enhance the tumor-inhibitory effect of an otherwise weakly inhibitory mAb. Another interesting observation relates to the target epitopes. Our most effective mAb combinations always included an antibody directed to the dimerization arm of HER2, a region permitting HER2 to form heterodimers with EGFR and ErbB-3. Whether these observations can be generalized and applied to tumor markers other than HER2 is an intriguing issue, the elucidation of which requires additional investigation and broader repertoires of mAbs to HER2. The current challenge is to identify pathway-specific therapies and explore their potential additive or preferably synergistic effects, while avoiding excessive toxicities.

Our study was recently extended to human pancreatic carcinoma, a malignancy with extremely poor prognosis, which is largely considered incurable. We compared the effects of nine homo- and hetero-combinations of mAbs to EGFR or HER2, on the growth of human pancreatic carcinoma BXPC3 expressing moderate level of EGFR and low level of HER2. MAbs to the two receptors inhibited tumor growth in animals as single agents but acted in synergy and were more effective when paired in homo-combinations, exerting improved inhibition. Anti-HER2 mAbs, despite the low HER2 receptor, acted as important partners in collaborating with mAbs to EGFR to form highly inhibitory pairs. These hetero-combinations acted in synergy and were the most effective in generating long-term inhibitory activity.

The low effectiveness of therapeutic mAbs and the evolution of patient resistance call for deeper understanding of mechanisms that underlay immunotherapy. Because the superiority of mAb combinations extends to tumor cell cultures, it may be assumed that in addition to cellular responses, non-immunological mechanisms also contribute to antibody synergy. Translation of these lessons to clinical applications may enhance patient response and delay acquisition of resistance.

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КОНЦЕПЦІЯ ВИКОРИСТАННЯ СИНЕРГІЇ МОНОКЛОНАЛЬНИХ АНТИТІЛ ДЛЯ СТВОРЕННЯ ЛІКАРСЬКИХ ПРЕПАРАТІВ

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Розглянуто історію відкриття явища синергічності моноклональних антитіл, результати досліджень протипухлинної активності їх різних комбінацій проти рецепторів тирозинкіназного EGFR/ErbB-1 і HER2/ErbB-2, а також фактора росту VEGF. Висловлено припущення про можливі молекулярні механізми, що лежать в основі явища синергічності моноклональних антитіл (для випадків гомо- і гетерокомбінацій антитіл, специфічних відповідно до антигенних детермінант одного й того самого або двох різних рецепторів). Обговорено напрями подальших досліджень, необхідних для глибшого розуміння причин цього явища, а також перспективи практичного застосування імунотерапевтичних препаратів на основі синергічних моноклональних антитіл для лікування пухлин людини.

Ключові слова: імунотерапія пухлин, моноклональні антитіла, синергічність, рецептори епідермальних факторів росту EGFR/ErbB-1 і HER2/ErbB-2, ендотелію судин (VEGF).

КОНЦЕПЦИЯ ИСПОЛЬЗОВАНИЯ СИНЕРГИИ МОНОКЛОНАЛЬНЫХ АНТИТЕЛ ДЛЯ СОЗДАНИЯ ЛЕКАРСТВЕННЫХ ПРЕПАРАТОВ

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Рассмотрены история открытия явления синергичности моноклональных антител, результаты исследований противоопухолевой активности их различных комбинаций против рецепторов тирозинкиназных EGFR/ErbB-1 и HER2/ErbB-2, а также фактора роста VEGF. Высказаны предположения о возможных молекулярных механизмах, лежащих в основе явления синергичности моноклональных антител (для случаев гомо- и гетерокомбинаций антител, специфичных соответственно к антигенным детерминантам одного и того же либо двух различных рецепторов). Обсуждены направления дальнейших исследований, необходимых для более глубокого понимания причин этого явления, а также перспективы практического применения иммунотерапевтических препаратов на основе синергических моноклональных антител для лечения опухолей человека.

Ключевые слова: иммунотерапия опухолей, моноклональные антитела, синергичность, рецепторы эпидермальных факторов роста EGFR/ErbB-1 и HER2/ErbB-2, эндотелия сосудов (VEGF).

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ПОЛУЧЕНИЕ МОНОКЛОНАЛЬНЫХ АНТИТЕЛ К ХОЛЕРНОМУ ТОКСИНУ И ТЕРМОЛАБИЛЬНОМУ ЭНТЕРОТОКСИНУ *E. coli* ДЛЯ РАЗРАБОТКИ БИПЛЕКСНОГО АНАЛИЗА ТОКСИНОВ В ОБЪЕКТАХ ОКРУЖАЮЩЕЙ СРЕДЫ

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Получены моноклональные антитела к холерному токсину и термолабильному энтеротоксину *E. coli*, перекрестно не взаимодействующие с родственным токсином. Подобраны пары антител для количественного определения этих токсинов в «сэндвич»-варианте иммуноэнзимного анализа и биплексном иммунофлуоресцентном анализе с применением технологии xMAP (Luminex). Минимальные детектируемые концентрации холерного токсина и термолабильного энтеротоксина *E. coli*, определенные в формате «сэндвич»-иммуноэнзимного анализа, — 0,2 и 0,4 нг/мл, значительно выше соответствующих величин в формате биплексного анализа — 0,01 нг/мл и 0,08 нг/мл. Присутствие в пробах молока, бульона и воды из открытого водоема, а также носоглоточных смывов не оказывает влияния на минимально детектируемые величины концентраций холерного токсина, определенные обоими методами. В аналогичных пробах, содержащих термолабильный энтеротоксин, они возрастали по сравнению с соответствующими для контрольного буфера.

Ключевые слова: иммуноэнзимный анализ, «сэндвич»-анализ, мультиплексный иммунофлуоресцентный анализ, xMAP-анализ, моноклональные антитела, минимальная детектируемая концентрация, холерный токсин, термолабильный энтеротоксин *E. coli*.

Интенсивное развитие международной экономической деятельности, открытие границ, усиливающаяся миграция населения, расширение сообщения и торговли привели к быстрому распространению инфекционных болезней. В современных условиях в любое время может начаться эпидемия, возбудителями которой могут быть новые или уже хорошо известные, занесенные из эндемических очагов, микроорганизмы. К основным современным источникам биологической опасности, представляющим наибольшую угрозу для населения, относят патогенные микроорганизмы, микроскопические грибы и продукты их жизнедеятельности. Токсические продукты микроорганизмов обладают высокой активностью и чрезвычайно токсичны для человека. В России имеется большой организационный опыт в области противодействия инфекционным заболеваниям, однако дальнейшее совершенствование методов диагностики и профилактики опасных инфекций на сегодняшний день остается важнейшей проблемой.

Бактериальные токсины — это секретируемые микробные протеины, обычно энзимы, которые повреждают и убивают клетки хозяина в исключительно низких концентрациях. Исходя из механизма действия токсины группируют следующим образом: порообразующие токсины (альфа-токсин *St. aureus*, гемолизин *E. coli*), ингибиторы синтеза протеина (дифтерийный токсин,

шига-токсин), генераторы образования вторичных мессенджеров (холерный токсин и термолабильный энтеротоксин *E. coli*), протеолитические токсины (ботулинические, столбнячный), активаторы иммунного ответа (стафилококковые энтеротоксины), трехсоставные токсины (сибиреязвенный токсин) [1].

Холерный токсин (CT), продуцируемый *Vibrio cholerae* и отвечающий за развитие симптомов такого смертельно опасного заболевания, как холера, и термолабильный энтеротоксин, продуцируемый энтеротоксигенными штаммами *E. coli* (LT), обусловливающий развитие симптомов менее опасного, чем холера, но более распространенного заболевания под названием «диарея путешественников», являются родственными токсинами. Они имеют большое сходство по структуре, функциональному действию, иммунохимическим характеристикам. В частности, сходство аминокислотной последовательности молекул токсинов составляет более 80% [2]. Поэтому большинство методов детекции не позволяют дифференцированно определять CT и LT в исследуемых образцах. Поскольку холера по сравнению с диареей путешественников при отсутствии лечения в 50% случаев заканчивается летальным исходом, большое значение в диагностике этих заболеваний имеет дифференцированное определение токсинов.

Холерный токсин, продуцируемый *V. cholerae*, относится к группе токсинов, генерирующих образование вторичных мессенджеров, способных в значительной степени усиливать и искажать клеточную реакцию на различные сигналы. Холерный токсин обладает АДФ-рибозилтрансферазной активностью по отношению к ГТФ-азе клеток млекопитающих. После АДФ-рибозилирования ГТФ-аза вызывает пролонгированную активацию аденилатциклазы и резкое увеличение цАМФ в энтероцитах. Увеличение концентрации цАМФ приводит к выведению ионов хлора, бикарбоната и воды из клеток, обусловливая тем самым потерю электролитов и сильное обезвоживание [3].

Указанные токсины традиционно детектируют биологическим методом, наблюдая их патогенное действие на одноклеточных организмах, культурах клеток, куриных эмбрионах, а также на лабораторных животных. Эти методы трудоемки, длительны (около 2–6 сут) и характеризуются высокой себестоимостью. Для тестирования одной пробы используют несколько животных, тесты проводят только в лабораториях, имеющих специальный сертификат [4, 5]. Для детекции и количественного определения токсинов используют также инструментальные методы, основанные на применении оборудования с высокой чувствительностью детекции: масс-спектрометрию, высокоэффективную жидкостную хроматографию, капиллярный электрофорез и т. д. По чувствительности эти методы сравнимы с биологическими, но их использование ограничено кругом специально оборудованных лабораторий, имеющих в своем распоряжении дорогостоящую аппаратуру и высококвалифицированные кадры, что делает использование этих методов недоступным широкому кругу специалистов [6].

В настоящее время наиболее широкое распространение в практике диагностирования бактериальных токсинов получили методы иммуноанализа. Ключевым компонентом в иммуноаналитических системах являются антитела. Для иммунохимического анализа токсинов используют три основных типа антител: моноспецифические поликлональные, моноклональные и рекомбинантные антитела или фрагменты антител [7].

Цель настоящего исследования — получение методом гибридомной техники моноклональных антител к холерному токсину и термолабильному энтеротоксину *E. coli*, не обладающим перекрестной активностью; определение их иммунохимических характеристик и создание биплексной тест-системы на основе мультиплексного иммунофлуоресцентного анализа с применением микросфер (МИА) для одновременной количественной детекции холерного токсина и термолабильного энтеротоксина *E. coli* в одной анализируемой пробе.

Экспериментальная часть

В работе использовали следующие реактивы: среда Игла в модификации Дульбекко (DMEM), фетальная телячья сыворотка (FCS), глутамин, раствор гипоксантин-аминоптерин-тимидин (HAT) — (GIBCO-Invitrogen, США); неполный адъювант Фрейнда, пристан, BSA, DMSO, 4-хлор-1-нафтол, 3,3-диаминобензидин, OPD (ортофенилендиамин), *N*-оксисукцинимидный эфир биотина, Твин-20, метилизотиазолона гидрохлорид (MIT) и холерный токсин (*Vibrio cholerae*) — Sigma (США), SDS — Serva (Германия), полиакриламид — Amresco (США); нитроцеллюлозная мембрана BA 85 — Schleicher & Schuell (Германия); протеин–А — сефароза —

GE-Healthcare (США), поликлональные козьи антитела, меченные пероксидазой хрена, к мышиным иммуноглобулинам, меченный пероксидазой хрена стрептавидин и набор для изотипирования иммуноглобулинов (mouse immunoglobulin isotyping ELISA kit) — BD Biosciences Pharmingen (США); культуральный пластик и планшеты для ИЭА — Costar (США); карбоксилированные xMAP микросферы — Luminex Corporation (США), 1-этил-3-(3-диметиламинопропил)-карбодиимид гидрохлорид (EDC), *N*-гидроксисульфосульфосукцинимид (*sulfo*-NHS) — Pierce (США); стрептавидин, меченный фикоэритрином (SA-PE) — One Lambda (США), поликлональные антитела, меченные фикоэритрином, к мышиным иммуноглобулинам — Dako (Швеция), снятое сухое молоко — Fluka (Германия). Стафилококковый энтеротоксин A (SEA), стафилококковый энтеротоксин B (SEB), термолабильный энтеротоксин *E. coli* (LT) были предоставлены д. б. н. Ю. В. Вертиевым (Институт эпидемиологии и микробиологии им. Гамалеи РАМН, Москва).

Получение гибридом

Гибридомы, продуцирующие моноклональные антитела к холерному токсину и термолабильному энтеротоксину *E. coli*, получали согласно общепринятому методу гибридомной технологии, предложенному Келлером и Мильштейном [8]. Для иммунизации использовали мышей (самки весом 18 г) линии BALB/c категории SPF и конвенциональной категории. Животных иммунизировали в подушечки задних лапок препаратом токсина в присутствии неполного адъюванта Фрейнда.

Мышей категории SPF иммунизировали CT или LT в дозе 10 мкг/мышь дважды с интервалом 2 недели. Мышей конвенциональной категории иммунизировали CT или LT трижды с интервалом 2 нед дозами токсина от 2,5, 5 и 20 мкг/мышь. На 4-й день после 2-й иммунизации в сыворотке крови мышей обеих категорий определяли методом непрямого твердофазного ИЭА титр специфических антител к CT и LT.

Через 6 сут после последней иммунизации у мышей забирали подколенные лимфоузлы, из которых выделяли лимфоциты для гибридизации с клетками миеломы SP2/0. Гибридизацию проводили при соотношении клеток 5:1 в 45%-м растворе ПЭГ-4000с, 10% DMCO в среде DMEM в течение 1 мин при совместной инкубации. После гибридизации клетки разносили по 96-луночным планшетам, в которые за 24 ч до этого помещали мышиные макрофаги. Культивировали клетки в селективной среде следующего состава: DMEM с 20% FCS, 4 мМ *L*-глутамином, 5 мМ меркаптоэтанолом, 0,1 мМ гипоксантином, $1{,}6\cdot10^{-5}$ М тимидином, $4\cdot10^{-7}$ М аминоптерином.

После завершения этапа селекции гибридных клеток от родительской миеломы их культивировали в среде, не содержащей аминоптерин. Через 7–10 сут после гибридизации из лунок с активно растущими клетками отбирали культуральную среду и тестировали ее непрямым твердофазным ИЭА на наличие антител к холерному токсину и термолабильному энтеротоксину *E. coli*. Из лунок, в культуральной среде которых регистрировали достоверно положительную реакцию с токсинами, отбирали клетки и клонировали их трижды методом предельных разведений в HT-среде роста. После двух последних клонирований число положительных клонов обычно составляло 100%.

Продукция антител в асцитной жидкости мышей

Мышам линии Balb/C (самки) вводили внутрибрюшинно 0,5 мл пристана. Через 10 дней животным вводили суспензию клеток гибридом в количестве $(0{,}5–1)\cdot10^{7}$ клеток. После формирования асцита мышей декапитировали, забирали асцитную жидкость, содержащую моноклональные антитела, и центрифугировали на 300 g в течение 10 мин при комнатной температуре. Клеточный осадок подвергали процедуре замораживания при −70 °C, а из супернатанта выделяли моноклональные антитела.

Очистка моноклональных антител из асцитной жидкости аффинной хроматографией на протеин А-сефарозе

На колонку, содержащую протеин А-сефарозу, объемом 3 мл, уравновешенную 5 объемами стартового буфера (1,5 М глицин, 0,15 мМ NaCl, pH 8,9), наносили образец — асцитную жидкость (3 мл), разведенную тремя объемами стартового буфера. Колонку промывали стартовым буфером, собирая в отдельную пробирку не связавшиеся с сорбентом протеины. Затем элюировали протеины буфером для элюции (0,1 М цитрат Na) с уменьшением градиента pH от 6,0 до 4,0. Наличие протеинов в элюатах регистрировали на проточном спектрофотометре при длине волны 280 нм. Регенерацию колонки проводили в 0,1 М цитратном буфере, pH 3,0.

Все образцы, элюированные с колонки отдельными фракциями, диализовали против PBS с добавлением 0,02% азида натрия при 4 °С в течение ночи. Препарат антител, полученных после хроматографии на протеин А-сефарозе, концентрировали методом ультрафильтрации с использованием ячеек для ультрафильтрации (Amicon, США) и мембран, отсекающих протеины с молекулярной массой более 10 кДа, до достижения концентрации антител в растворе 1–1,5 мг/мл. Концентрацию выделенного протеина определяли спектрофотометрически при длине волны 280 нм.

Электрофорез в полиакриламидном геле

Чистоту полученных препаратов антител оценивали электрофоретически. Электрофорез проводили в восстанавливающих условиях в 12,5% ПААГ по методу Лэммли [9]. К анализируемому препарату антител добавляли равный объем 2-кратного разделяющего буфера, pH 6,8, содержащего 0,125 М Tris-HCl, 4% SDS и 20%-й глицерол, 10%-й 2-меркаптоэтанол. Образцы нагревали на водяной бане в течение 3 мин. В каждый образец добавляли по 1 мкл 0,2%-го раствора бромфенолового синего и вносили в лунки полиакриламидной пластинки, состоящей из концентрирующего 4%-го геля и разделяющего 12,5%-го геля. В качестве маркеров использовали смесь очищенных стандартных протеинов: фосфорилаза В (97кДа), альбумин (66 кДа), овальбумин (45 кДа), карбоангидраза (30 кДа), ингибитор трипсина (20,1 кДа) и α-лактальбумин (14,4 кДа). Электрофорез проводили при силе тока 10 мА до полного введения бромфенолового синего в гель, а затем при 20 мА. После окончания процесса (1,5–2 ч) гель окрашивали в течение 30 мин в 0,125%-м растворе Кумасси R-250 с последующим отмыванием геля в обесцвечивающем растворе до полного исчезновения фоновой окраски.

Тестирование специфической активности антител к холерному токсину и термолабильному энтеротоксину E. coli методом непрямого твердофазного ИЭА

Для тестирования использовали коммерческий препарат CT с исходной концентрацией 2 мг/мл, препарат LT с исходной концентрацией 0,32 мг/мл. Аликвоту исходных препаратов разводили в 0,1 М бикарбонатном буфере, pH 9,0, до концентрации 1 мкг/мл. Приготовленные растворы по 100 мкл вносили в лунки ИЭА-планшетов с высокой степенью связывания. Планшеты инкубировали в течение ночи при 4 °С. После инкубации свободные центры связывания на пластике планшетов блокировали 1%-м раствором BSA при 37 °С в течение 1 ч. Лунки планшетов промывали 3 раза PBS с 0,05%-м Твин-20 (PBST) и 3 раза — PBS. После промывания вносили 100 мкл культуральной среды или препаратов очищенных антител и планшеты инкубировали в течение 1 ч при 37 °С. По окончании времени инкубации поверхность лунок планшета отмывали (как описано выше), вносили пероксидазный конъюгат кроличьих антител против иммуноглобулинов мыши, инкубировали 40 мин при комнатной температуре, отмывали и добавляли субстрат пероксидазы — OPD в концентрации 1 мг/мл в 50 мМ цитратном буфере, pH 4,5, содержащем 0,015% пероксида водорода. После развития окраски реакцию останавливали добавлением 50 мкл 10%-й серной кислоты. Интенсивность окраски регистрировали спектрофотометрически, определяя оптическое поглощение при длине волны 492 нм.

Определение константы аффинности антител к холерному токсину и термолабильному энтеротоксину E. coli

Константу аффинности моноклональных антител при связывании с холерным токсином или с термолабильным энтеротоксином *E. coli* определяли по методу Битти непрямым твердофазным ИЭА [10].

Токсины сорбировали на поверхности лунок ИЭА-планшета с высокой степенью связывания из растворов, взятых в двух концентрациях 0,5 мкг/мл и 1 мкг/мл, в объеме 100 мкл на лунку. Далее проводили непрямой твердофазный ИЭА, как описано выше. Препараты анализируемых антител титровали от 40 мкг/мл до 40 пг/мл с шагом 2. Строили графики зависимости оптического поглощения при длине волны 492 нм от концентрации МА. Константу аффинности определяли по формуле:

$$K_{\text{афф}} = 1/(4[AT'] - 2[AT]) \pm 3 \cdot SD,$$

где [AT′] — концентрация антител, соответствующая 50%-му связыванию при внесении в лунку планшета токсина в концентрации 0,5 мкг/мл; [AT] — концентрация антител, соответствующая их 50%-му связыванию при внесении в лунку токсина в концентрации 1 мкг/мл; *SD* — среднее квадратичное отклонение от среднего арифметического значения A_n, $n = 3$.

Получение конъюгатов биотина с моноклональными антителами к холерному токсину и термолабильному энтеротоксину E. coli

К препарату очищенных антител (1 мг/мл) в 0,1 М бикарбонатном буфере, pH 9,0, добавляли раствор оксисукцинимидного эфира биотина в ДМСО (1 мг/мл) из расчета 120 мкл реагента на 1 мг протеина. Смесь инкубировали в темноте 3 ч при комнатной температуре, добавляли 1 М раствор трисглицинового буфера, pH 8,2, в объеме 50 мкл на 1 мл антител и диализовали против PBS. Полученный препарат биотинилированных антител хранили при 4 °С в PBS с 0,02% азидом Na.

«Сэндвич»-вариант ИЭА в формате планшета

В лунки ИЭА-планшета с высокой степенью связывания вносили 100 мкл раствора связывающих антител к CT или LT в PBS с концентрацией 10 мкг/мл и инкубировали планшет в течение ночи при 4 °С. Свободные центры связывания на поверхности лунок планшета блокировали 1%-м раствором сухого молока инкубацией в течение 1 ч при 37 °С. Далее в лунки планшета вносили токсин (CT или LT) в концентрации 1мкг/мл и делали двоичные и троичные разведения токсина в PBST (от 1 мкг до 0,1 нг/мл), инкубировали в течение 1 ч при 37 °С, затем планшет отмывали 3 раза раствором PBST и 3 раза раствором PBS. В пробу добавляли по 100 мкл проявляющих меченных биотином МА к соответствующему токсину (10 мкг/мл) и инкубировали в течение 1 ч при 37 °С. После отмывки добавляли раствор стрептавидина, меченного пероксидазой хрена, разведенного 1:1 000 в PBST, и инкубировали 40 мин при 37 °С. В качестве субстрата пероксидазы использовали раствор OPD (1 мг/мл). После развития окраски реакцию останавливали добавлением 50 мкл 10%-й серной кислоты. Интенсивность окраски регистрировали спектрофотометрически, определяя оптическое поглощение при длине волны 492 нм.

Минимальную детектируемую концентрацию токсина определяли по:

$$\overline{A}_0 + 3 \cdot SD,$$

где $\overline{A}_0$ — среднее значение величин оптической плотности в лунках, не содержащих токсин; *SD* — среднее квадратичное отклонение от среднего арифметического значения A_0.

SD вычисляли по формуле:

$$SD = \sqrt{\frac{\sum_{i=1}^{n} (A_0 - \overline{A_0})^2}{n-1}},$$

где A_0 — среднее арифметическое значение оптической плотности в лунках, не содержащих токсин; n — число измерений. В большинстве экспериментов $n = 3$.

Получение конъюгатов моноклональных антител к холерному токсину и термолабильному энтеротоксину E. coli с микросферами

Конъюгирование связывающих антител с микросферами проводили согласно предложенному компанией Luminex протоколу для конъюгации таких реагентов с помощью sulfo-NHS/EDC.

Для конъюгации со связывающими антителами к CT применяли микросферы с номером ID 29, а для конъюгации со связывающими антителами к LT — микросферы с номером ID 36. Количество конъюгированных микросфер подсчитывали микроскопически с использованием камеры Горяева.

Анализ эффективности конъюгации микросфер со связывающими антителами к холерному токсину и термолабильному энтеротоксину E. coli

Эффективность конъюгации микросфер со связывающими антителами оценивали по величине интенсивности медианного флуоресцентного сигнала, регистрируемого в спектральной области соответствующего ID используемых микросфер.

Препарат микросфер, конъюгированных с различными количествами (1, 5 и 25 мкг) антител, суспендировали и обрабатывали ультразвуком в течение 20 с. Полученную (стоковую) суспензию микросфер разводили до концентрации $6 \cdot 10^5$ микросфер в 1 мл и вносили в лунки планшета с фильтрующим дном MABVIN1250 plate (Millipore, США). Антимышиные антитела, меченные фикоэритрином, разводили PBS-BSA до концентрации 6 мкг/мл. Из препарата готовили троичные разведения, каждый разведенный препарат вносили в лунки, содержащие микросферы, конъюгированные с антителами. После инкубации супернатант из всех лунок одновременно удаляли при помощи вакуумного насоса со специальной насадкой для планшетов с фильтрующим дном (Millipore, США). После промывок микросферы суспендировали PBS для анализа на проточном анализаторе Luminex 200.

Подбор оптимальных концентраций биотинилированных (детектирующих) антител для количественного определения холерного токсина и термолабильного энтеротоксина E. coli методом xMAP-анализа

Препарат микросфер, конъюгированных с различными количествами (1, 5 и 25 мкг) связывающих антител к CT или LT, суспендировали и обрабатывали ультразвуком как описано выше. В полученной суспензии концентрацию микросфер доводили до $1{,}8 \cdot 10^5$ в 1 мл и помещали по 30 мкл в лунки планшета с фильтрующим дном. Далее в лунки добавляли 30 мкл токсина в концентрациях 30, 300 и 3 000 пг/мл. В эти же лунки вносили по 30 мкл соответствующих биотинилированных антител в концентрациях 0,5; 1,0; 2,0 и 4,0 мкг/мл. Планшет инкубировали в течение 1 ч при постоянном перемешивании в темноте при комнатной температуре. После инкубации лунки планшета промывали трижды PBS-BSA. В лунки планшета вносили по 100 мкл коммерческого препарата стрептавидина, меченного фикоэритрином. Планшет инкубировали в течение 30 мин при постоянном перемешивании в темноте при комнатной температуре. По окончании времени инкубации лунки планшета промывали трижды PBS-BSA и однократно PBS. После промывок микросферы суспендировали в 100 мкл PBS и анализировали на проточном лазерном анализаторе Luminex 200.

Определение минимальной детектируемой концентрации холерного токсина и термолабильного энтеротоксина E. coli в мультиплексном «сэндвич»-xMAP-анализе

Препараты микросфер, конъюгированные со связывающими антителами к CT и LT суспендировали и обрабатывали ультразвуком. Суспензии микросфер, конъюгированные со связывающими антителами к CT и LT, смешивали таким образом, чтобы в общем объеме концентрация микросфер каждого типа составляла $6 \cdot 10^5$ микросфер/мл. Полученную суспензию обоих типов микросфер помещали по 50 мкл в лунки планшета с фильтрующим дном. Холерный токсин и термолабильный энтеротоксин *E. coli* смешивали таким образом, чтобы в общем объеме PBS-BSA их концентрация составляла 320 и 800 пг/мл соответственно. Делали двоичные разведения в круглодонном планшете и вносили соответствующие разведения токсинов по 50 мкл в лунки, содержащие микросферы. Планшет инкубировали при постоянном перемешивании в темноте в течение 1 ч при комнатной температуре. Далее лунки планшета промывали трижды PBS-BSA. В лунки вносили по 100 мкл раствора, содержащего биотинилированные антитела к LT и CT, разведенные в PBS-BSA в оптимальных концентрациях (2 мкг/мл для обоих токсинов). Планшет инкубировали при постоянном перемешивании в темноте в течение 1 ч при комнатной температуре. По окончании времени инкубации лунки планшета промывали трижды и добавляли по 100 мкл коммерческого препарата стрептавидина, меченного фикоэритрином. Последующие стадии инкубации, отмывки и анализа на проточном лазерном анализаторе проводили так же, как при подборе оптимальных концентраций биотинилированных (детектирующих) антител для количественного определения холерного токсина и термолабильного энтеротоксина *E. coli* методом xMAP-анализа.

Минимальную детектирующую концентрацию определяли как самую низкую, с флуоресцентным сигналом, превышающим более чем на три стандартных отклонения среднее значение фонового флуоресцентного сигнала. Стандартное отклонение среднего значения фонового сигнала вычисляли по формуле:

$$SD = \sqrt{\frac{\sum_{i=1}^{n}(A_0 - \overline{A_0})^2}{n-1}},$$

где $\overline{A}_0$ — среднее арифметическое значение медианного флуоресцентного сигнала в лунках, не содержащих токсин; n — число измерений; $n = 3$.

Подготовка образцов для определения в них токсинов методом «сэндвич»-ИЭА в формате планшета и xMAP-анализа

Для определения CT и LT в биологических образцах препараты токсинов разводили BSA-PBS до концентрации 1 мкг/мл. Полученные растворы разводили образцами молока, мясного бульона, воды, носоглоточных смывов, не содержащими токсинов, до концентрации 1 нг/мл для CT и 10 нг/мл для LT. Образцы с токсинами подвергали центрифугированию при 5 000 *g* в течение 5 мин. Для анализа отбирали интерфазу, переносили в лунки круглодонного планшета и делали двоичные и троичные разведения (CT — от 1 до 0,01 нг/мл, LT — 10 до 0,1 нг/мл). Для xMAP-анализа подготовленные образцы, содержащие токсины, переносили по

50 мкл в лунки, содержащие конъюгаты связывающих антител с микросферами. Далее определяли токсины согласно вышеприведенному методу.

Для определения CT или LT в воде, молоке и мясном бульоне методом «сэндвич»-ИЭА в формате планшета 100 мкл токсина в различных концентрациях (от 1 мкг до 0,1 нг/мл) вносили в лунки планшета и инкубировали в буфере, содержащем 10% воды из открытого водоема, коммерческого молока или мясного бульона. Далее проводили определение согласно методу «сэндвич»-варианта ИЭА в формате планшета, описанному выше.

Для получения образца смыва со слизистой носоглотки человека использовали тампон из синтетического негигроскопичного материала. С помощью тампона собирали со слизистой носоглотки биоматериал. Тампон тщательно отмывали в 1 мл рабочего раствора PBST для «сэндвич»-ИЭА или в 1 мл BSA-PBS для МИА, центрифугировали при комнатной температуре при 5 000g в течение 5 мин и анализировали на содержание токсинов.

Результаты и обсуждение

Успех в получении гибридом — продуцентов моноклональных антител — определяется прежде всего эффективной схемой иммунизации животных (обычно мышей) — доноров иммунных лимфоцитов, являющихся партнерами при слиянии с клетками миеломы. Были выбраны короткие схемы иммунизации, инициирующие развитие локального иммунного ответа. Для получения гибридом, продуцирующих моноклональные антитела к холерному токсину и термолабильному энтеротоксину *E. coli*, использовали клетки миеломы SP2/0 — плазмоцитомной клеточной линии, не секретирующей иммуноглобулины, и лимфоциты, выделенные из подколенных лимфоузлов мышей линии BALB/c конвенциональной категории с естественной микрофлорой и категории SPF (specific pathogen free), свободных от патогенной микрофлоры [11]. Мышей иммунизировали токсином CT (Sigma) и LT, выделенным из клеток *E. coli* штамм YM107, трансформированных плазмидой pLT, содержащий ген с полной последовательностью LT. Токсины вводили в подушечки задних лапок в присутствии неполного адъюванта Фрейнда (НАФ). Основанием для такого выбора адъюванта послужила имеющаяся в литературе информация о том, что холерный токсин, а также термолабильный энтеротоксин *E. coli* обладают ярко выраженными свойствами адъюванта [12, 13]. Агентом, способствующим соматической гибридизации клеток, выбран полиэтиленгликоль 4 000.

Такие схемы иммунизации не обеспечивали очень высоких титров специфических антител в сыворотке крови, однако формирование лимфоцитов — предшественников плазматических клеток в лимфоузлах происходило в достаточной степени для получения гибридом, стабильно продуцирующих антитела в высоких титрах в культуральной жидкости, где титр составлял не менее 1:20 000. Отбор жизнеспособных гибридом проводили по стандартной схеме в культуральной среде, содержащий НАТ [8]

Скрининг клонов-продуцентов осуществляли методом непрямого ИЭА. Для CT и LT уже на первом этапе отбора целевых клонов был осуществлен перекрестный скрининг с целью исключения из дальнейшей работы клонов гибридом, способных продуцировать антитела к общим антигенным детерминантам этих токсинов. После проведения первого скрининга клоны гибридом, продуцирующие специфические антитела к CT или LT, подвергали клонированию методом последовательных разведений и повторному скринингу. Число клонов, продуцирующих антитела, которые детектировали оба токсина, было значительным и составляло более 30% от общего количества. В результате гибридизации лимфоцитов мышей конвенциональной категории, иммунизированных LT, было получено 7 гибридных клонов, а мышей категории SPF — 8 клонов, стабильно продуцирующих специфические МА, не связывающиеся с CT. После гибридизации лимфоцитов мышей конвенциональной категории, иммунизированных CT, получили 14 клонов, не детектирующих LT, а вследствие гибридизации лимфоцитов мышей категории SPF — 20 клонов [11].

Характеристика МА к CT и LT включала изотипирование и определение типа тяжелых и легких цепей иммуноглобулинов. Изотипирование проводили в соответствии с рекомендацией к коммерческому набору для изотипирования мышиных иммуноглобулинов. Результаты представлены в табл. 1 и 2. Здесь же приведены величины констант аффинности антител $K_{афф}$, установленные согласно методу Битти [10]. Величины констант аффинности МА к CT варьируют от $0{,}11 \cdot 10^9$ M^{-1} до $2{,}9 \cdot 10^9$ M^{-1}; МА к LT — от $0{,}16 \cdot 10^9$ M^{-1} до $2{,}0 \cdot 10^9$ M^{-1}. Для определения

констант использовали высокоочищенные препараты антител, выделенные из асцитной жидкости мышей, привитых клетками гибридом.

Таблица 1. **Характеристика моноклональных антител к термолабильному энтеротоксину *E. coli***

Гибридный клон	Тип тяжелой цепи	$К_{афф}$ 10^9 $М^{-1}$
E11F4	IgG1	0,16 ±0,02
D11H11	IgG1	0,24±0,03
2F4D9	IgG1	0,4 ±0,04
G2H4	IgG1	0,27±0,02
F5G2	IgG1	1,5 ±0,04
F8F6	IgG1	1,8±0,04
1B1G6F2	IgG3	0,5±0,02
D12B9	IgG1	0,3±0,01
D2E4	IgG1	0,3±0,02
B11G11	IgG1	2,0±0,04

Таблица 2. **Характеристика моноклональных антител к холерному токсину [8]**

Клон	Типы тяжелой цепи	$К_{афф}$ 10^9 $М^{-1}$
A2B1	IgG1	0,42 ± 0,04
A6B9	IgG3	0,53 ± 0,04
A7G3	IgG3	0,21 ± 0,02
A11E3	IgG2a	0,74 ± 0,06
B1F8	IgG1	0,25 ± 0,04
C2B4	IgG3	2,2 ± 0,1
C5H10	IgG1	1,1 ± 0,1
C7F11	IgG3	2,9 ± 0,2
C10F10	IgG1	0,12 ± 0,02
D3D8	IgG2a	1,4 ± 0,1
D4D6	IgG2b	0,33 ± 0,03
D5C3	IgG3	1,2 ± 0,1
D6G7	IgG2b	0,11 ± 0,01
D7F11	IgG1	0,22 ± 0,02
E3B6	IgG1	0,13 ± 0,02
E6E10	IgG1	0,52 ± 0,03
F4F4	IgG1	1,3 ± 0,4
F5H3	IgG1	2,6 ± 0,1
G6D12	IgG1	0,62 ± 0,04
G7D8	IgG1	2,7 ± 0,15
H4B6	IgG1	0, 51 ± 0,03
H5E10	IgG1	0,35 ± 0,04
H5G8	IgG3	0,45 ± 0,05
H8F8	IgG3	0,30 ±0,02

Как следует из приведенных в табл. 1 и 2 данных, все моноклональные антитела относятся к иммуноглобулинам класса G, подклассам IgG1, IgG2a, IgG2b и IgG3, что обеспечило определенные преимущества в выборе метода их очистки с помощью иммуноаффинной хроматографии на протеин А-сефарозе. Все эти антитела имели легкую цепь к-типа. В результате очистки выделены электрофоретически чистые препараты антител (рис. 1), пригодные для получения их биотинилированных производных.

Рис. 1. **Электрофореграмма моноклональных антител к CT B1F8 и к LT F5G2 (дорожка *1* и *2* соответственно) в 12,5% ПААГ–SDS в восстанавливающих условиях.**
На дорожке *3* представлена электрофореграмма смеси стандартных протеинов: фосфорилаза В (97 кДа), альбумин (66 кДа), овальбумин (45 кДа), карбоангидраза (30 кДа), ингибитор трипсина (20,1 кДа) и α-лактальбумин (14,4 кДа)

Одним из методов выявления бактериальных токсинов в аналитических концентрациях является «сэндвич»-вариант ИЭА. В этом формате высокая чувствительность достигается применением двух МА-связывающих и детектирующих антител к различным эпитопам молекулы токсина. Связывающие антитела иммобилизованы на поверхности лунок планшета, а детектирующие представляют собой меченые биотином антитела. Для мечения детектирующих МА использовали сукцинимидный эфир биотина.

С целью подбора пары антител, позволяющей в «сэндвич»-ИЭА выявлять CT и LT с высокой чувствительностью, все полученные антитела (табл. 1 и 2) тестировали в качестве как связывающих, так и детектирующих антител. Для этого каждое из антител было биотинилировано. Таким образом, все антитела были представлены в нативном виде и в виде биотинилированных производных.

Из полученных спектров антител подбирали пары, дающие возможность выявлять холерный токсин или термолабильный энтеротоксин *E. coli* с МДК менее 1 нг/мл. Такая величина МДК соответствует пределу детекции коммерческой тест-системы CT и LT VET-RPLA detection kit (Oxoid) [14]. Отобранные пары МА в отсутствие токсина не связывались друг с другом и, таким образом, не формировали в пробах существенного неспецифического фона. Эти пары антител (табл. 3 и 4) не взаимодействовали с LT или CT соответственно.

Для определения чувствительности «сэндвич»-варианта ИЭА детекции CT или LT в буфере использовали двоичные и троичные разведения токсинов в интервале концентрации от 1 мкг/мл до 0,1 нг/мл. Минимальную детектируемую концентрацию (МДК) токсинов определяли как концентрацию, соответствующую значению оптического поглощения, превышающего не менее чем на три стандартных отклонения оптическое поглощение многократно измеренной нулевой точки (в отсутствие токсина). Пары антител оценивали по соответствующим величинам МДК.

Таблица 3. **Пары моноклональных антител, выявляющие в «сэндвич»-анализе холерный токсин в концентрации ниже 1 нг/мл [8]**

Связывающие антитела	Детектирующие антитела	МДК CT, нг/мл	Связывающие антитела	Детектирующие антитела	МДК CT, нг/мл
B1F8	B1F8biot	0,4	F5/H3	B1F8biot	0,2
D4D6	D6G7biot	0,4		E6E10biot	0,2
	E6E10biot	0,4		D6G7biot	0,2
D7F11	D6G7biot	0,4	G7D8	D6G7biot	0,4
	B1F8biot	0,4		B1F8biot	0,4
F4F4	E6E10biot	0,4		E6E10biot	0,4
	B1F8biot	0,8	H8F8	D6G7biot	0,4
H4B6	D6G7biot	0,4		E6E10biot	0,4

Таблица 4. **Пары моноклональных антител, выявляющие в формате «сэндвич»-ИЭА термолабильный энтеротоксин *E. coli* с чувствительностью ниже 1 нг/мл**

Связывающие антитела	Детектирующие антитела	Нижний предел детекции LT, нг/мл	Связывающие антитела	Детектирующие антитела	Нижний предел детекции LT, нг/мл
E11F4	F5G2biot	0,4	E11F4	B11G11biot	1,1
2F4D9		0,4	D11H11		0,6
G2H4		0,8	G2H4		0,4
F8F6		1,1	F5G2		0,5
1B1G6F2		0,3	F8F6		0,8
D12B9		0,9	1B1G6F2		0,6
B11G11		0,4	D12B9		0,9
E11F4	1B1G6F2biot	0,4	D2E4		0,5
2F4D9		0,9	B11G11		0,5
G2H4		0,6	E11F4	G11F12biot	0,5
F5G2		0,4	2F4D9		0,4
F8F6		0,5	G2H4		0,4
1B1G6F2		0,6	F5G2		1,0
B11G11		0,5	F8F6		0,4

Как следует из представленных в табл. 3 данных, пары антител F5H3–D6G7biot, F5H3–E6E10biot и F5H3–B1F8biot выявляли СТ с нижним пределом детекции 0,2 нг/мл токсина. При использовании антител D6G7, E6E10, B1F8 в качестве связывающих и антител F5H3 как детектирующих МДК СТ увеличивалась и составляла более 1 нг/мл холерного токсина. Следует отметить, что константа аффинности для антител F5H3 (табл. 2) была значительно выше, чем для D6G7, E6E10 и B1F8, что, очевидно, определило позицию МА F5H3 в качестве связывающего в «сэндвич»-анализе [11].

По данным табл. 4, тест-система определения LT методом «сэндвич»-ИЭА при использовании антител F5G2, 1B1G6F2, B11G11 и G11F12 в качестве детектирующих характеризовалась наименьшими величинами МДК. Эти антитела составляли пары со многими связывающими антителами и определяли LT с минимальными значениями МДК (менее 1 нг/мл). Антителам F5G2, 1B1G6F2, B11G11 и G11F12 присущи самые высокие константы аффинности (табл. 1) порядка 10^9 M^{-1}. Минимальный предел детекции LT составил 0,4 г/мл для пар B11G11–F5G2biot, E11F4–F5G2biot, F5G2–B11G11biot, D2E4–B11G11biot и B11G11–B11G11biot соответственно. В этих парах связывающие антитела также имели высокие константы аффинности.

Разработанная тест-система позволяет детектировать СТ и LT в меньшей концентрации, чем МДК коммерческой тест-системы VET-RPLA detection kit (Oxoid) [10], используемой для детекции СТ и LT методом непрямой пассивной латексной агглютинации, позволяющий определять энтеротоксины в концентрации 1–2 нг/мл. Следует отметить отсутствие перекрестного связывания МА к СТ с термолабильным энтеротоксином *E. coli* (LT), а антител к LT — с холерным токсином. Значения МДК известной иммуноэнзимной тест-системы дифференцированной детекции СТ и LT с применением пар, составленных из моноклональных и поликлональных антител, составляет 0,1 нг/мл [15]. Нами получены пары, составленные из моноклональных антител, позволяющие детектировать СТ и LT в буфере с МДК, сравнимой с МДК вышеназванной тест-системы — 0,2 нг/мл для СТ и 0,4 нг/мл для LT.

Полученные высокочувствительные и моноспецифические МА к LT и СТ определили возможность разработки биплексной иммунофлуоресцентной тест-системы на основе xMAP-технологии.

Разработанная тест-система на основе xMAP-технологии является разновидностью «сэндвич»-варианта метода ИХА, в котором связывающие антитела иммобилизованы на твердом носителе — полистирольных гранулах (микросферы) диаметром 5,6 мкм. Применяемые в этом методе микросферы уникальны по спектральным характеристикам, обусловленным комбинацией внутренних флуорофоров. Детектирующие антитела — вторые из подобранной пары — мечены фикоэритрином. Детекцию флуоресцентной метки проводили, используя компактный проточный флуоресцентный анализатор (Luminex 200) с программным обеспечением для обработки результатов Luminex IS 2.3. [16].

При разработке биплексной тест-системы на первом этапе работы исследовали пригодность отобранных методом «сэндвич»-ИЭА пар МА в xMAP-анализе. Разрабатывая тест-систему на основе xMAP-анализа, использовали 3 пары антител к СТ (F5H3–B1F8biot, F4F4–B1F8biot, F4F4–E6E10biot) и 5 пар антител к LT (E11F4–F5G2biot, E11F4–1B1G6F2biot, E11F4–B11G11biot, F5G2–1B1G6F2biot и F5G2–B11G11biot) [16].

Ключевой стадией в xMAP-технологии является получение высокоэффективного конъюгата микросфер со связывающими антителами. Связывающие МА к токсинам иммобилизовали на поверхности карбоксилированных полистирольных частиц карбодиимидным способом. При разработке тест-системы для определения холерного токсина использовали карбоксилированные микросферы xMAP 129, имеющие спектральный адрес (ID) 29. В качестве связывающих СТ антител применяли F5H3 и F4F4. Для определения LT использовали карбоксилированные микросферы xMAP 136 (ID36). Связывающими LT антителами служили МА E11F4 и F5G2.

Для каждого МА подбирали оптимальные условия конъюгации. С этой целью конъюгацию микросфер проводили с различными количествами МА (25, 5 и 1 мкг). Эффективность процесса конъюгации микросфер с различными количествами связывающих антител оценивали по интенсивности медианного флуоресцентного сигнала полученного комплекса с поликлональными кроличьими антимышиными иммуноглобулинами, меченными фикоэритрином. Каждая анализируемая проба характеризовалась значениями интенсивности медианного флуоресцентного сигнала (MFI), представляющего собой среднее значение интенсив-

ности флуоресценции 100 анализированных микросфер. Для выявления конъюгата микросфер со связывающими антителами с максимальной интенсивностью медианного флуоресцентного сигнала изучали зависимость сигнала от различных концентраций поликлональных кроличьих антимышиных меченных фикоэритрином иммуноглобулинов.

Максимальную интенсивность флуоресценции при минимальном неспецифическом фоне наблюдали для микросфер, конъюгированных с антителами F4F4 и F5H3 в количестве 1 и 5 мкг, а для антител E11F4 и F5G2 — 1 мкг.

Проведение экспериментов по определению оптимального количества связывающих антител, конъюгированных с микросферами, является обязательным для характеристики эффективности конъюгации.

На следующем этапе работы для отобранных конъюгатов связывающих антител с микросферами осуществляли подбор оптимальных концентраций детектирующих антител, меченных биотином.

В экспериментах использовали четыре концентрации биотинилированных антител — 0,5; 1,0; 2,0; 4,0 мкг/мл и три концентрации токсина — 30, 300 и 3000 пг/мл. Максимальную интенсивность флуоресценции при минимальном неспецифическом фоне наблюдали при концентрации детектирующих антител B1F8 2 мкг/мл, для антител E6E10 — 4 мкг/мл, F5G2 — 2 мкг/мл, а для 1B1G6F2 — 1 мкг/мл.

В табл. 5 приведены оптимальные количества связывающих антител, использованных в конъюгации с микросферами ID29 и ID36, и оптимальные концентрации детектирующих антител, определяющие максимальную интенсивность медианного флуоресцентного сигнала.

Определяли минимальную детектируемую концентрацию токсинов в буфере PBS-BSA в индивидуальном и биплексном анализах. Микросферы с иммобилизованными на них специфическими МА к CT и LT инкубировали с соответствующими токсинами в различных концентрациях. МДК определяли в соответствии с общепринятыми стандартами как концентрацию, соответствующую среднему уровню флуоресцентного сигнала аналита, превышающему среднее значение фонового сигнала флуоресценции на три стандартных отклонения. В табл. 6 приведены величины МДК CT и LT.

Самое низкое значение МДК для CT (10 пг/мл) было определено с использованием следующих антител: связывающие анти-

Таблица 5. **Пары МА, отобранные для xMAP-анализа токсинов CT и LT**

Токсин	Область ID	Связывающие антитела		Детектирующие антитела	
		Клон	Количество антител, иммобилизованных на микросферах, мкг	Клон	Концентрация биотинилированных антител, мкг/мл
CT	36	F4F4	1	B1F8	2
			5	E6E10	4
		F5H3	1	B1F8	2
LT	29	E11F4	1	F5G2	2
				1B1G6F2	1
		F5G2	1	1B1G6F2	1

Таблица 6. **Минимальные детектируемые концентрации CT и LT, определенные в xMAP-анализе с участием различных пар связывающих антител, иммобилизованных на микросферах, и детектирующих антител**

Токсин	Антитела, конюгированные с микросферами	Детектирующие антитела, несущие биотиновую метку	МДК, пг/мл
CT	F5F3	B1F8	10
	F4F4	E6E10	20
	F4F4	B1F8	10
LT	E11F4	F5G2	80
	E11F4	1B1G6F2	160
	F5G2	1B1G6F2	100

тела F4F4, конъюгированные с микросферами и работающие в паре с детектирующими антителами B1F8biot, и связывающие антитела F5H3, конъюгированные с микросферами, в паре с детектирующими B1F8biot. В дальнейшей работе использовали пару антител F4F4 с биотинилированными B1F8. При определении МДК термолабильного энтеротоксина *E. coli* наибольшая чувствительность показана для связывающих антител E11F4, конъюгированных с микросферами, работающими в паре с детектирующими F5G2biot.

Для указанных выше пар с минимальными значениями МДК CT и LT был проведен биплексный анализ, результаты которого представлены на рис. 2.

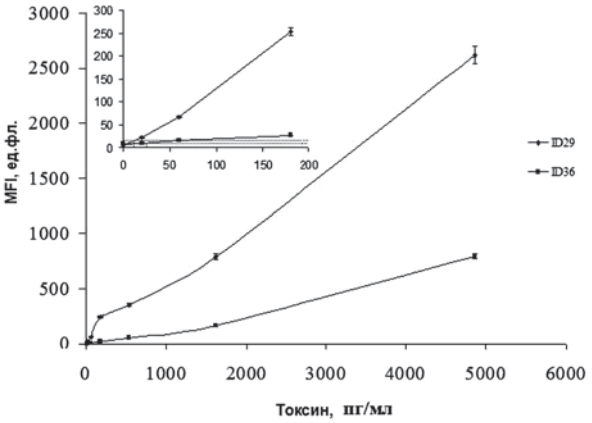

Рис. 2. **Зависимость интенсивности медианного флуоресцентного сигнала от концентрации CT и LT в xMAP-анализе, проведенном с участием пулов микросфер, конъюгированных с F4F4 и с E11F4, и с пулами детектирующих антител B1F8 biot и F5G2 biot.**

На врезке приведен калибровочный график, соответствующий области низких концентраций токсинов. Пунктирной линией обозначено значение флуоресцентного сигнала, превышающее среднее значение флуоресцентного сигнала нулевой пробы на три стандартных отклонения. Концентрация CT и LT при этих значениях флуоресцентного сигнала соответствует минимальной детектируемой концентрации

Из приведенных данных следует, что МДК CT превышает МДК LT, что согласуется с данными, полученными при xMAP-анализе этих двух токсинов в индивидуальном анализе. В биплексном формате показатели, соответствующие фоновому сигналу флуоресценции, не превышали аналогичные показатели, полученные при xMAP-анализе токсинов в индивидуальном анализе. В табл. 7 представлены значения МДК для CT и LT, регистрируемые в тест-системах формата xMAP-анализа и «сэндвич»-ИЭА.

Из данных таблицы следует, что МДК токсинов, определенные в биплексном и индивидуальном анализах, не различаются, однако они существенно ниже величин МДК токсинов, установленных с помощью «сэндвич»-ИЭА в формате планшета.

Было проведено сравнительное определение МДК токсинов LT и CT в модельном буфере и в различных образцах объектов окружающей среды, продуктах питания и носоглоточных смывах человека методами «сэндвич»-ИЭА в формате планшета и иммунофлуоресцентном анализе с применением xMAP-технологии. Результаты представлены в табл. 8.

Из представленных данных следует, что чувствительность определения холерного токсина в молоке, бульоне, воде и в смывах носоглотки сравнима с чувствительностью определения токсина в модельном буфере. Значения МДК в биплексном анализе на два порядка превышают аналогичные значения, определенные в «сэндвич»-ИЭА. Для LT наблюдается увеличение значений МДК в молоке и в бульоне по сравнению с величиной в контрольном буфере. МДК, определенные в воде и в смывах со слизистой носоглотки человека, сравнимы с МДК в контрольном буфере. Значения МДК LT в биплексном анализе превышают, в основном на порядок, соответствующие величины МДК, полученные методом «сэндвич»-ИЭА. Эта мультиплексная тест-система позволяет определять токсины и в различных биологических

Таблица 7. **Минимальные концентрации холерного токсина и термолабильного энтеротоксина *E. coli*, детектируемые в биплексном иммунофлуоресцентном анализе с применением xMAP-технологии и в «сэндвич»-ИЭА в формате планшета**

Токсин	МДК токсина в индивидуальном анализе, нг/мл	МДК токсина в мультиплексном анализе, нг/мл	МДК токсина в «сэндвич»-ИЭА, нг/мл
CT	0,01	0,01	0,2
LT	0,08	0,08	0,4

Таблица 8. **Минимальные детектируемые концентрации (нг/мл) холерного токсина и термолабильного энтеротоксина *E. coli* в буфере, молоке, бульоне, смывах со слизистой носоглотки человека и в воде из открытого водоема, определенные методами «сэндвич»-ИЭА в формате планшета и биплексного анализа с применением xMAP-технологии**

Токсин		PBS-BSA	Молоко	Бульон	Вода	Носоглоточные смывы
МДК CT	ИФА	0,2	0,2	0,2	0,2	0,30
	xMAP	0,01	0,02	0,02	0,02	0,09
МДК LT	ИФА	0,4	9,6	6,8	2,0	8,0
	xMAP	0,08	3,0	1,0	0,1	0,8

жидкостях, в объектах окружающей среды и в продуктах питания. Содержание CT в молоке, мясном бульоне, в смывах с носоглотки можно устанавливать с такой же чувствительностью, как и для модельного буфера.

Таким образом, биплексная иммунофлуоресцентная тест-система на основе xMAP-технологии для одновременной детекции CT и LT создана нами в России впервые.

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ОДЕРЖАННЯ МОНОКЛОНАЛЬНИХ АНТИТІЛ ДО ХОЛЕРНОГО ТОКСИНУ І ТЕРМОЛАБІЛЬНОГО ЕНТЕРОТОКСИНУ *E. coli* ДЛЯ РОЗРОБЛЕННЯ БІПЛЕКСНОГО АНАЛІЗУ ТОКСИНІВ У ОБ'ЄКТАХ НАВКОЛИШНЬОГО СЕРЕДОВИЩА

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Отримано моноклональні антитіла до холерного токсину і термолабільного ентеротоксину *E. coli*, які перехресно не взаємодіють зі спорідненим токсином. Підібрано пари антитіл для кількісного визначення цих токсинів у «сендвіч»-варіанті імуноензимного аналізу і біплексному імунофлуоресцентному аналізі із застосуванням технології xMAP (Luminex). Мінімальні детектовані концентрації холерного токсину і термолабільного ентеротоксину *E. coli*, що їх визначено у форматі «сендвіч»-імуноензимного аналізу — 0,2 і 0,4 нг/мл, значно вищі за відповідні величини у форматі біплексного аналізу —0,01 нг/мл і 0,08 нг/мл. Присутність у пробах молока, бульйону та води з відкритого водоймища, а також носоглоткових змивів не справляє впливу на величини мінімально детектованих концентрацій холерного токсину, які визначено обома методами. В аналогічних пробах, що містять термолабільний ентеротоксин, вони зростали порівняно з відповідними показниками для контрольного буфера.

Ключові слова: імуноензимний аналіз, сендвіч-аналіз, мультиплексний імунофлуоресцентний аналіз, xMAP-аналіз, моноклональні антитіла, мінімальна концентрація, що детектується, холерний токсин, термолабільний ентеротоксин *E. coli*.

OBTAINING OF MONOCLONAL ANTIBODIES AGAINST CHOLERA TOXIN AND HEAT LABILE ENTEROTOXIN OF *E. coli* FOR DEVELOPMENT OF THE TOXINS DIPLEX ANALYSIS IN ENVIRONMENTAL SPECIMENS

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The present study focuses on development of monoclonal antibodies (MAbs) which specifically interact with cholera toxin or heat labile enterotoxin of *E. coli*. Such monoclonal antibodies MAbs are possessed of ability to identify cholera toxin or heat labile enterotoxin in different immunochemical assays. We obtained hybridoma clones which produced monoclonal antibodies of IgG isotypes to cholera toxin and heat labile enterotoxin. On application of the method of serial dilutions we selected the clones which produced monoclonal antibodies with specific activity against only one of the toxins. We found the 16 pairs of monoclonal antibodies to cholera toxin and 28 ones to heat labile enterotoxin. By means of these monoclonal antibodies it was possible to realize the quantitative analysis of theses toxins in sandwich immunoassay ELISA and diplex sandwich xMAP-assay. The limits of detection of cholera toxin and heat labile enterotoxin in ELISA in control buffer were 0.2 and 0.4 ng/ml, respectively, and in xMAP assay — 0.01 and 0.08 ng/ml, respectively. In probes of cow milk, meat soup, pond water and nasopharyngeal washes cholera toxin was detected in the both assays with the same limits of detections, but heat labile enterotoxin limits of detections were above the ones in control buffers.

Key words: ELISA, sandwich assay, multiplex immunoassay, xMAP analysis, monoclonal antibodies, minimal detectable concentration, cholera toxin, heat labile enterotoxin of *E. coli*.

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USAGE OF MONOCLONAL ANTIBODIES FOR DETERMINATION OF LOCALIZATION OF ANTIGENIC DETERMINANTS AND FIBRIN POLYMERIZATION SITES WITHIN FIBRINOGEN AND FIBRIN MOLECULES AND THEIR APPLICATION IN TEST-SYSTEMS FOR DIAGNOSTICS AND THE THREAT OF THROMBUS FORMATION

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It was shown by monoclonal antibodies that BβN-region of fibrin desA molecule (Bβ1-53) comprises the polymerization site including the peptide bond Bβ14-15. This site participates in the second stage of fibrin polymerization — lateral association of protofibrils. In the Bβ15-53 fragment was also found the site called «C», which together with the site «A» participate in the first stage of polymerization — the protofibrils formation. The model of the primary intermolecular interaction of fibrin was designed. It was found by monoclonal antibodies II-4d the site («c») in the N-terminal half of γ chain of the fibrin D-region. This site participates in the protofibrils formation and is complement to site «C» as we assume.

We have discovered two neoantigenic determinants. One of these determinants exposes within the coiled-coil fragment Bβ126-135 of fibrin as a result of fibrinopeptide A splitting off from fibrinogen by thrombin. The structural rearrangements discovered in this site of the fibrin molecule are necessary for the following protofibrils lateral association. The second neoantigenic determinant is localized in the fragment Bβ134-190 of D-dimer formed after plasmin degradation of fibrin stabilized by FXIIIa. We have obtained the fibrin-specific monoclonal antibodie FnI-3C to the first determinant and D-dimer-specific mAb III-3b to the second one.

Three monoclonal antibodies were obtained against the αC-region of fibrin(ogen) molecule. It has been experimentally shown by of one of them that αC-domains is connected with the fibrinopeptides B in fibrinogen and fibrin desA molecules, but removes from the core of the molecules after fibrinopeptides B splitting off by thrombin. Two other monoclonal antibodies specifically inhibit the fibrin polymerization by blocking two unknown polymerization sites within the αC-region.

The test-systems for the soluble fibrin and D-dimer quantification in human blood plasma were designed on the basis of monoclonal antibodies FnI-3C and III-3b as «catch»-antibodies and one II-4d as a «tag»-antibody, respectively. The clinical trials of the test-systems were carried out in Ukraine. It was shown that for the prediction of postoperative thrombotic complications and monitoring the efficiency of antithrombotic therapy the simultaneous quantification of soluble fibrin and D-dimer before the operation and at different time intervals after the operation is required. Only in this case it is possible to get information about the state of the balance between blood coagulation and fibrinolytic systems, and determine the degree of the threat of thrombosis.

Key words: fibrinogen, fibrin, monoclonal antibodies, thrombosis, diagnostic test systems.

Fibrinogen (Fg) is a protein playing the main role in functioning of blood coagulation system [1]. It has a dimeric structure where two subunits are formed by three polypeptide chains: Aα, Bβ and γ. The fibrinogen molecule consists of a central E, two peripheral D-regions and two extended αC-regions.

The E-region, consisting of (Aα1-104, Bβ1-133, γ1-72)$_2$, is formed by the N-terminal segments of all six polypeptide chains. The two D-regions, each comprising of Aα105-219, Bβ134-461 and γ73-411 in the C part of the molecule, have the globular βC232-461 and γC170-411 nodules. The extended αC-regions

(Aα220-610) consist of an unstructured flexible αC-connector (Aα220-391) and a more structured αC-domain (Aα392-610) [2, 3].

The peripheral D-regions are connected with the central E-region by two long flexible coiled-coil connectors each of which consists of polypeptide fragments Aα48-161, Bβ79-193, γ23-135. The N-parts of these fragments include Aα48-104, Bβ79-133, γ23-62 and belong to the E-region. The C-parts Aα105-161, Bβ134-193, γ63-135 belong to the D-region (Fig. 1).

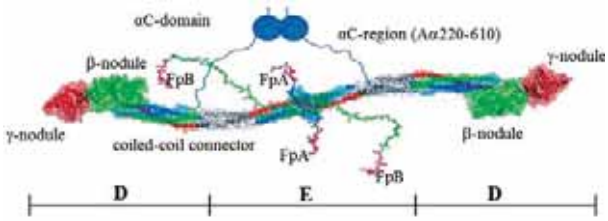

Fig. 1. **The model of fibrinogen molecule created with graphics system PyMol on the base of X-ray and NMR analysis of fibrin(ogen) fragments [2].** Aα-chains — blue; Bβ-chains — green; γ-chains — red

The splitting off of fibrinopeptides A (FpA) — Aα1-16 from fibrinogen (Fg) by thrombin results in the formation of desA fibrin (Fn) with two exposed polymerization sites called A-knobs [4]. The two A-knobs interact with a-holes in the two D-regions of the other Fn molecules forming protofibrils. The protofibrils associate laterally, giving rise to fibrils and, finally, the three-dimensional fibrin net. At the stage of protofibril and fibril formation thrombin splits off of fibrinopeptides B (FpB) — Bβ1-14 from Fn desA molecules exposing the B-knobs which are involved in the process of protofibril lateral association interacting with the b-holes in the D-regions of the other molecules [5].

Two steps of fibrin polymerization — protofibrils formation and their lateral association — are carried out by the intermolecular and interprotofibril binding of specific polymerization sites [6, 7]. Polymeric fibrin is stabilized by factor XIIIa [8]. After polymeric fibrin formation plasminogen is activated on fibrin surface by tPA and transformed into plasmin [9, 10]. Plasmin splits polymeric fibrin stabilized by factor XIIIa forming E-fragment and D-dimer.

Monoclonal antibody (mAb) can be obtained against antigenic determinant (epitope), which can coincide or be located closely to the polymerization site of fibrin molecule. Such a mAb presumably inhibits fibrin polymerization by blocking the polymerization site or the neighboring one. MAb can also «recognize» minor alterations in the epitope conformation. That is why mAbs have been used as molecular probes for localization of unknown antigenic determinants and/or polymerization sites [11, 12].

The tactics of our investigations was following:

- obtaining of antigen for mice immunization: native or slightly denaturated fibrin(ogen) molecules or their fragments;
- obtaining of mAbs;
- selection of mAbs and obtaining their Fab-fragments, which both specifically inhibit fibrin polymerization;
- electron microscopy for determination of fibrin polymerization stage, which is inhibited by the mAb and Fab-fragment.
- epitope localization in fibrin(ogen) molecule for mAb — inhibitor;
- synthesis of the peptide, which imitates the amino acid sequence of fibrin molecule in the monAb epitope region;
- testing of the synthetic peptide inhibitory action on fibrin polymerization.

Three types of monoclonal antibodies of different specificity have been obtained against the N-terminal disulphide knots of fibrinogen and fibrin. Their effects on distinct stages of fibrin polymerization have been studied. These antibodies were shown to be directed against various epitopes of the Bβl-53 fragment of the fibrinogen molecule. The mAbs had different effects both on the rate of protofibril lateral aggregation and on the final turbidity of fibrin clots. The mAbs were of three specificities: mAb 2d-2a and its Fab-fragment inhibited the rate of lateral aggregation of protofibrils and decreased the turbidity of the final clot; those from clone B-4C accelerated the polymerization, but did not affect the clot turbidity; and those from clone D-IB did not have any effect on either fibrin polymerization or final clot turbidity and served as the negative control. The most interesting results were obtained with mAb 2d-2a, epitope for which was localized in fibrin desA fragment including the peptide bond Bβ14-15 [13]. This mAb and its Fab-fragment inhibited the lateral association of fibrin desA protofibrils (Fig. 2) by blocking the site comprising amino acid residues located around the peptide bond Bβ14-15. We suggested that N-terminal part of fibrin desA Bβ-chain concludes one of the active sites involved in protofibril lateral

association. Fibrinopeptide B does not need to be split off for functioning of this site. Interestingly that only one mAb molecule can bind to one of two its epitopes in dimeric fibrin desA molecule blocking one peptide bond Bβ14-15 and inhibiting 60% of fibrin polymerization. In such a case, thrombin may split off only one fibrinopeptide B from fibrinogen. However, two Fab-fragments of this mAb can bind to both epitopes in fibrin desA, blocking them and inhibiting about 100% of polymerization. This mAb has been used as catch-antibody in our ELISA test-system for fibrinogen quantification in human blood plasma after its dilution in 200 times [14].

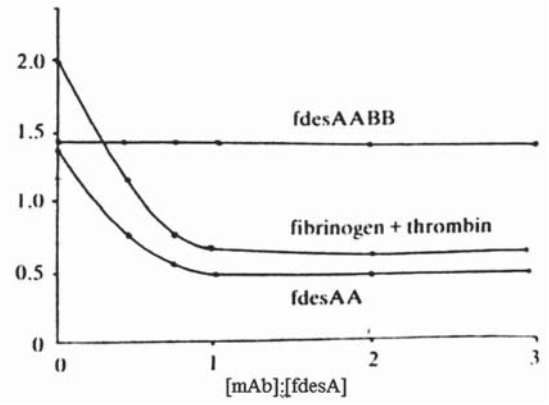

Fig. 2. **Dependence of the rate of protofibrils lateral association (V) on the concentration of mAb 2d-2a**

D-dimer of human fibrin was also used as antigen to obtain monoclonal antibodies. We have obtained 16 hybridomas producing mAbs of different specificity. MAb III-3b binds D-dimer with $K_d = 1.4 \cdot 10^{-10}$ M without cross-reaction with fibrinogen and fibrin. The epitope for this mAb is located in fibrin(ogen) fragment Bβ134–190 (Fig. 3). The latter site is buried in the coiled-coil structure of fibrin(ogen), but it is exposed as a neoantigenic determinant (NAD) in D-dimer upon plasmin

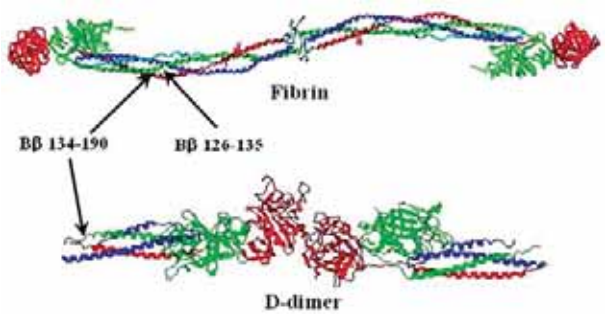

Fig. 3. **The scheme of epitopes localization for mAb I-3c and mAb III-3b in fibrin and D-dimer molecules, respectively**

hydrolysis of fibrin [15]. MAb III-3b has been used in our ELISA immunodiagnostic test-system as a catch-antibody for quantification of D-dimer in human blood plasma [16].

Only two of these 16 hybridomas produced mAbs of the IgG-class that inhibited fibrin polymerization. MAb II-4d inhibited fibrin polymerization to 100% at a molar ratio mAb:fibrin = 1.0. Fab-fragments of the mAb inhibited fibrin polymerization completely at the same molar ratio (Fig. 4, *1*). The epitopes for the mAbs studied are situated in the NH_2-terminal part of the γ-chain in fibrin D-domain. Electron microscopy showed that fibrin was in monomeric form in the presence of these mAbs or their Fab-fragments (Fig. 4, *2*). Thus, these mAbs inhibit the initial step of fibrin polymerization, i.e. protofibril formation. Only one site of protofibril formation located in COOH-terminal half of the D-domain γ-chain is known now named «a» site, which is complementary to the «A» site in the central E-domain of fibrin molecule. Our experiment with immobilized GPRP showed that the «a» site in fibrin D-fragment preserved its binding activity to GPRP when the D-fragment was complexed with mAbs-inhibitors of fibrin polymerization. Thus, these two mAbs inhibit fibrin polymerization not by blocking the sites «a», but either by blocking another (inconsistent with «a») unknown specific site of polymerization in D-domain or by steric hindrance of highly organized fibrin polymerization process [17]. MAb II-4d has been used in three our immunodiagnostic test-systems for fibrinogen, soluble fibrin and D-dimer quantification in human blood plasma as the tag-antibody [14, 16, 18]

Four mAbs of the IgG_1 class to the thrombin-treated N-terminal disulfide knot of fibrin, secreted by various hybridomas, have been selected. Epitopes for two mAbs I-3C and

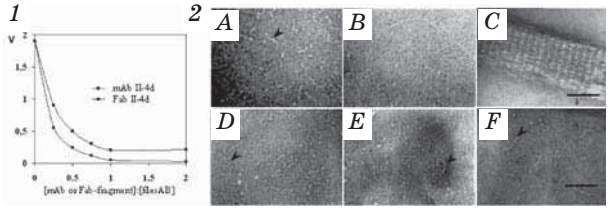

Fig. 4. 1 **— Dependence of the velocity of fibrin polymerization (V) on the molecular ratio of mAb II-4d and it Fab-fragments to fibrin desAB. *2* — Electron microscope images of fibrin desAB polymerizing in the presence (*A, B, C*) and in the absence (*D, E, F*) of mAb II-4d after 3, 5 and 7 min, correspondingly. There were no difference between electron microscope images obtained in the presence of Fab-fragments and mAb II-4d**

III-10d were located in human fibrin fragment Bβ15–26, and epitopes for mAbs I-5G and I-3B were in fragment Bβ26–36. Three of these mAbs, I-5G, I-3B and III-10D as well as their Fab-fragments decreased the maximum rate of fibrin desA and desAB polymerization up to 90–95% at a molar ratio of mAb (or Fab-fragment) to fibrin of 1 or 2. The fourth mAb I-3C did not influence on fibrin desAB polymerization and inhibited by 50% the maximum rate of fibrin desA polymerization. These results suggest that these mAb-inhibitors block a longitudinal fibrin polymerization site. As the mAbs retard both fibrin desAB and fibrin desA polymerization, one can conclude that the polymerization site does not coincide with polymerization site 'B' (Bβ15-17). To verify this suggestion, the polymerization inhibitory activity of synthetic peptides BβSARGHRPLDKKREEA(12–26), BβLDKKREEA(19–26), BβAPSLRPAPPPI(26–36), BβAPSLRPAPPPISGGGYRARPA(26–46) and BβGYRARPA(40–46), which imitate the various sequences in the N-terminal region of the fibrin Bβ-chain, have been investigated. Peptides Bβ12–26 and Bβ26–46, but not Bβ40–46, Bβ19–26, and Bβ26–36, proved to be specific inhibitors of fibrin polymerization. The IC_{50} values for Bβ12–26 and Bβ26–46 were $2.03 \cdot 10^{-4}$ and $2.19 \cdot 10^{-4}$ M, respectively. Turbidity and electron microscopy data showed that peptides Bβ12–26 and Bβ26–46 inhibited the fibrin protofibril formation stage of fibrin polymerization. The conclusion was drawn that fibrin fragment Bβ12–46, named as the site «C», took part in fibrin protofibril formation simultaneously with site «A» (Aα17–19) prior to removal of fibrinopeptide B. A model of the intermolecular connection between fragment Bβ12–46 of one fibrin desA molecule and the D-domain of another has been constructed (Fig. 5) [7].

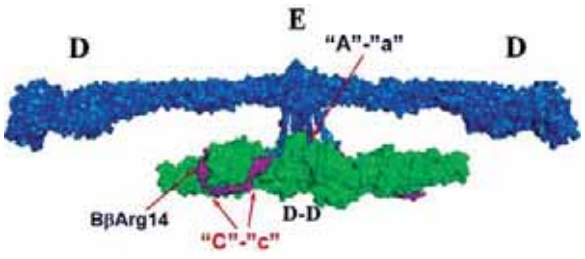

Fig. 5. **The model of the intermolecular connection between the D-domain of one fibrin desAA molecule (blue) and Bβ12–46 (magenta) of another. The model was prepared with PYMOL [17] on the basis of the X-ray analysis data of chicken fibrinogen [18] and human D-dimer bound with synthetic peptide GPRP [19]**

While the fibrin protofibril formation mechanism is well-studied, the protofibril lateral association mechanism remains elusive. The initial interaction of fibrin monomers leading to protofibril formation is realized mainly by the pair of complementary centers A:a. An additional site «C» of protofibril formation is localized within the E domain [7]. The protofibril lateral association is realized by the lateral interactions of the D-D dimers from the neighboring protofibrils. The interacting fragments were determined as γ 350-360 and γ 370-380. The interprotofibril D-D dimer interactions lead to formation of so called D-tetramers [19].

However, Kollman *et al* [20] have discovered antiparallel contacts of fibrinogen coiled-coil fragments in the crystals of human fibrinogen and proposed the possibility of intermolecular coiled-coil interactions during the fibrin protofibril lateral association. We obtained earlier [21] the fibrin-specific mAb FnI-3c, which reacts with fibrin and does not react with fibrinogen and D-dimer. The NAD of this mAb formed during fibrinogen to fibrin transformation was localized preliminary within the BβM118-V133 fragment, which is the part of the coiled-coil connector of the fibrin(ogen) molecule. It was shown that mAb I-3c and its Fab-fragment specifically inhibit the stage of fibrin protofibril lateral association (Fig. 6). Thus, we suggested that this region also participates in the process of the fibrin protofibril lateral association [21].

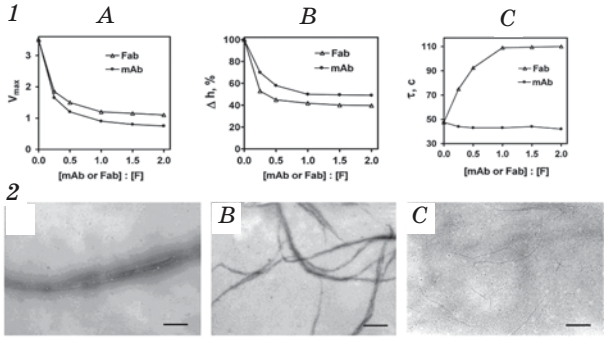

Fig. 6. **The influence of mAb FnI-3C and its Fab-fragment on polymerization of fibrin produced in fibrinogen+thrombin reaction in turbidity analysis (*1*). The dependence of the maximum rate of the fibrin polymerization V_{max} (*A*), final turbidity of fibrin clots Δh (*B*) and the lag time τ (*C*) on molar ratio of mAb FnI-3C or its Fab-fragment to fibrin. (*2*) Electron micrographs of negatively contrasted structures formed during polymerization of fibrin produced in fibrinogen+thrombin reaction in 135 s from start of the process: in the absence mAb FnI-3C or Fab-fragment (*A*); in the presence of mAb FnI-3C (*B*) or its Fab-fragment (*C*). The bars represent 200 nm**

Turbidity analysis and electron microscopy, showed that the synthetic peptide imitating fibrin fragment Bβ121-138, but not Bβ109-126, also inhibits the step of the fibrin protofibril lateral association. SPR analysis revealed that mAb FnI-3C interacts with the peptide Bβ121-138. This mAb also interacts with human, horse, and rabbit fibrins, but not with cow and rat fibrins. Alignment of the amino acid sequences of these fibrins allowed us to identify the residue BβK130 as crucial for the mAb FnI-3C epitope formation and to propose that the epitope in human fibrin is formed by the amino acid residues of the Bβ126-135 fragment. With the help of electrophoretic and ELISA parallel analysis we demonstrated that the exposition of the epitope is not the result of the removal of the αC-regions from the bulk of the molecule during the fibrinogen-fibrin transformation. SPR analysis reveals that the exposition takes place as the result of FpA splitting in a monomeric fibrin [22]. The analysis of the mutations in the of coiled-coil connector of the fibrin molecule corresponding to Bβ111-141, which impair fibrin polymerization, demonstrates that these mutations alter the structure of this fragment, but not its surface. Contrariwise, the mutations in the fragment Bβ158-170 of the coiled-coil connector alter the surface. The comparative conformational analysis of the fibrinogen and fibrin molecules suggests that the fragment of the coiled-coil connector corresponding to Bβ126-135 is more flexible in fibrin. The mobility of the fragments of the molecule around this fragment is higher in fibrin than in fibrinogen. Thus, we suppose that FpA splitting by thrombin leads to structural rearrangements in the human fibrin fragment of the coiled-coil connector, which includes polypeptide fragments Aα91-103, Bβ126-135 and γ69-77. These structural rearrangements provide fibrin protofibrils with a capability to associate laterally forming fibrils. The other fragment of the coiled-coil connector including Bβ158-170 may act as one of interprotofibril contact sites (Fig. 7).

MAb I-3C has been used as a «catch»-one in double-sandwich ELISA for soluble fibrin quantification in human blood plasma [18].

Monoclonal antibodies FnII-2M [23], I-5B and I-6B [24] to the αC-region of the human fibrin(ogen) have been obtained. The epitope for mAbs FnII-2M, I-5B are localized within the fibrin fragment Aα240-491 and for mAb I-6B — within Aα509-602 fragment. By virtue of the monAb FnII-2M, which react only with fibrin desAB and does not react with Fg and

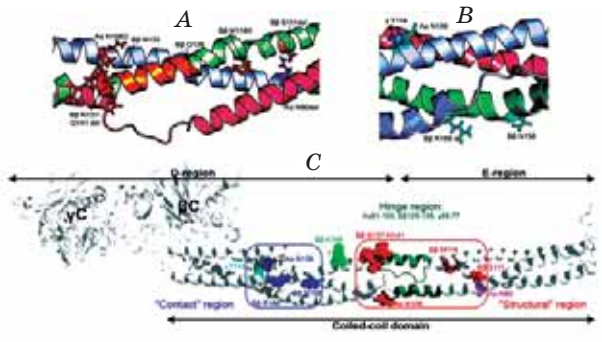

Fig. 7. A — **The localization of five human fibrinogen mutations in Bβ 111-141 and in the corresponding α-chain fragments that alter the structure of this coiled-coil fragment in mutant fibrinogens: Kyoto IV (Bβ S111del), Lyon (Bβ M118K), Epsom (Bβ N137- E141 del) and Plzen (Aα N106D) are shown as red sticks and Caracas VI (Aα N80del) is shown as magenta sticks. The suggested epitope for mAb FnI-3c (Bβ 126-135) is in orange.** ***B*** **— The localization of the amino acid residues where the structural alterations take place in the four mutant fibrinogens: Lima (Aα R141S), Niigata (Bβ N160S), Longmont (Bβ R166C) and San Diego II (γ Y114H) are shown as sticks. The novel sites Aα N139 and Bβ N158 of oligosaccharide attachment in fibrinogens Lima and Niigata are blue. The amino acid residue Bβ 166, where an individual cysteine from the medium attaches to Bβ 166C in fibrinogen Longmont (Bβ R166C), is blue. In fibrinogen San Diego II the buried hydrophobic Y114 (cyan) is replaced by H.** ***C*** **— The location of the SLA/NAD «structural» site corresponding to Bβ 111-141, the «contact» site corresponding to Bβ 158-170, and the «hinge region» (Aα 91-103, Bβ 126-135 and γ 69-77) in the human fibrin(ogen) molecule (PDB ID: 3GHG). The «structural» site and the hinge region are on the border between the D- and E-parts of the coiled-coil domain. The «contact» site is in the C- terminal part of the coiled-coil connector on the lateral side of the D-region of the molecule**

fibrin desA it was found that αC-regions are connected with FpB in the fibrinogen, monomer and polymer fibrin desA. However, the αC-regions move away from the core of the fibrin desA molecules after FpB cleavage by thrombin from fibrin desA in protofibrils, resulting in enhancing lateral association of protofibrils. It was been confirmed by ELISA and method of surface plasmon resonance (SPR) that monAb FnII-2M does not react with fibrinogen, monomeric and polymeric fibrin desA, but react with fibrin desAB. Thus, accessibility of this monAb to its epitope exists only in fibrin desAB. These results are direct experimental evidence that αC-regions (Aα220-610) are bound FpB in fibrinogen and fibrin desA molecules, but move away from the core of the molecule only after removal of

fibrinopeptides B (FpB) by thrombin. These data and the results of the other authors let us to design the three-dimensional structural computer models reflecting of αC-regions spatial orientation in fibrinogen, fibrin desA and fibrin desAB molecules (Fig. 8) [23].

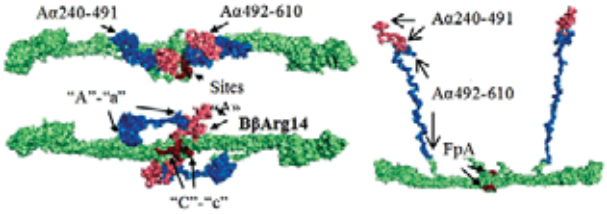

Fig. 8. **The models of fibrinogen, fibrin desA and fibrin desAB were done with PyMOL and Modeller9v10**

It was investigated that mAbs I-5B and I-6B decreased the maximum rate of fibrin desAB polymerization up to 90% and 76.2%, respectively, at their equimolar ratio to fibrin. These results suggest that these mAbs-inhibitors block the sites into αC-region, which take part in protein-protein interactions during the fibrin polymerization. The mAbs I-5B and I-6B may be used as tag-antibodies together with fibrin-specific «catch»-antibodies I- for quantification of the earliest forms of soluble fibrin in human blood plasma with the aim of early diagnostics of thrombophilia.

Soluble fibrin and D-dimer are the most specific markers of activation of the blood coagulation cascade and the threat of thrombosis. We have designed three immunoassay test-systems for D-dimer, soluble fibrin and fibrinogen quantification in human blood plasma on the basis of D-dimer-specific, fibrin-specific and fibrinogen-specific monoclonal antibodies. The forth test-systems was designed for simultaneous quantification of these molecular markers (Fig. 9). The clinical trials of the test systems were carried out in Ukraine. The high informativity of soluble fibrin quantification as a prognostic indicator of the threat of thrombosis at hip replacement (Fig. 10) and abdominal aorta (Fig. 11) was shown. Independent D-dimer quantification is uninformative. For the prediction of postoperative thrombotic complications and monitoring the efficiency of antithrombotic therapy the simultaneous quantification of soluble fibrin and D-dimer before the operation and at different time intervals after the operation is required. Only in this case it is possible to get information about the state of the balance between blood coagulation and fibrinolytic systems, and determine the degree of the threat of thrombosis.

Fig. 9. **The immunoassay test-systems for D-dimer, soluble fibrin and fibrinogen and its simultaneous quantification in human blood plasma designed by Palladin Institute of Biochemistry of NAS of Ukraine and LLC «Diaproph»**

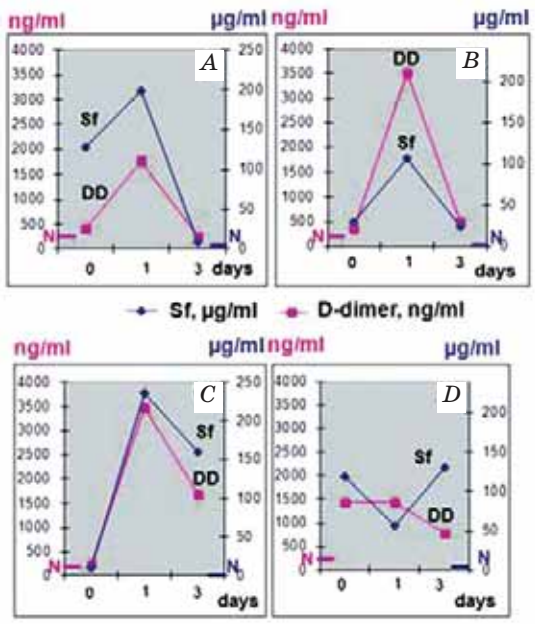

Fig. 10. **These figures show the typical combinations of the soluble fibrin (Sf) and D-dimer (DD) concentrations during aneurism of abdominal aorta before the operation, immediately after the operation and on the third day after the operation. Normal (*B, C*) or increased (*A, D*) concentrations of Sf and D-dimer were observed before the operation. After the operation in three cases (*A, B, C*) the significant increase of both markers was observed. On the third day after the operation in two of these cases (*A, B*) we can see the decrease of these concentrations to normal level. In the case (*C*) the concentrations of these markers also decreased, but remained above normal level, that required a further control. In the cases (*A, B, C*) there is a dynamic balance between blood clotting and fibrinolytic systems. It is illustrated by correlated concentration increase and decrease of both markers. In the case D: at once after the operation the concentration of Sf tended to decrease, and the concentration of D-dimer remained at the same level. But on the third day after the operation a steep increase of Sf and the decrease of D-dimer concentrations were observed. This suggests the disturbance of the balance between blood clotting and fibrinolytic systems and the high threat of thrombus formation**

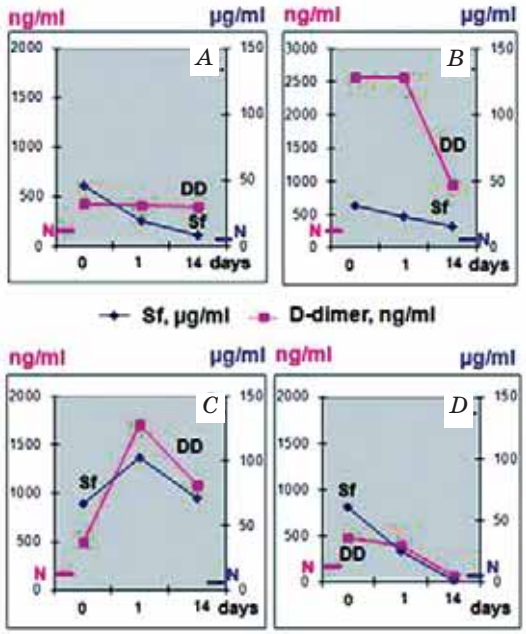

Fig. 11. **These figures show the typical combinations of the soluble fibrin (Sf) and D-dimer (DD) concentrations during deep vein thrombosis before the operation, immediately after the operation and on the 14 day after the operation. The main feature of this disease is high concentrations of both markers before treatment that indicates the high degree of activation of blood clotting and fibrinolytic systems. This suggests that during this pathology the activation of blood clotting system as a rule leads to the activation of fibrinolytic system. The dynamics of the concentration changes of these markers are usually correlated: we can see simultaneous increase (*C*) or decrease (*A, B, D*) of the concentrations of both markers during treatment. In the case (*C*) the correlated changes of the concentrations of these markers also takes place, but the high concentrations of these markers on the 14 day after operation testify about necessity of further control**

A hundred of mAbs against neoantigenic determinants of fibrinogen, fibrin and their fragments were obtained. In this review we focused on 14 of them, that were used to obtain the new fundamental scientific information or to develop immunodiagnostic test-systems, which have strategic importance for diagnostics of the threat of thrombus formation.

The arrows at the Fig. 12 point at the sites of fibrinogen and fibrin molecules, the mAbs are directed to. Thus, mAb 2d-2a was obtained against the epitope, which consists of amino acid residues located around the peptide bond Bβ14-15 cleaved by thrombin. This MAb reacted with fibrinogen and fibrin desA but doesn't with fibrin desAB. These molecules keep the fibrinopeptides B uncleaved. Using 2d-2a mAb we showed for the first time the existence of the site of fibrin protofibrils lateral association within the BβN-domain. This site functions before fibrinopeptides B cleavage off. This work was continued and 4 other mAbs against BβN-domain (Bβ1-64) were obtained. Two of them were directed to site Bβ15-25, and others to Bβ26-36. Using these mAbs we discovered that there is not only the site of the protofibrils lateral association in the region of BbN-domain, but also the site, which takes a part in the first stage of fibrin polymerization — protofibrils formation.

MAb III-3b obtained in this work reacts only with D-dimer, but doesn't with fibrinogen and fibrin. By virtue of this mAb test-system for quantification of D-dimer in human blood plasma was developed to diagnose the hemostasis disorders. Other MAb II-4d allowed us to locate the fibrin polymerization site in the NH_2-terminal part of γ-chain of D-region, which does not coincide with the

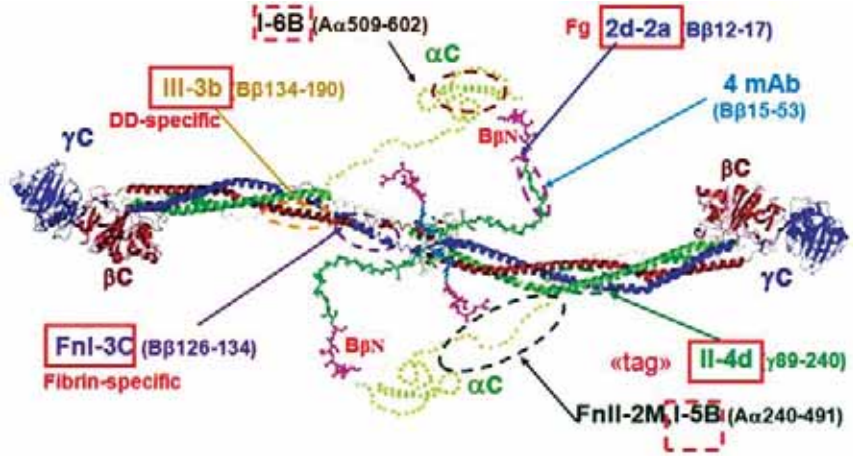

Fig. 12. **The model of fibrinogen molecule, where are shown the mAbs and localization of epitopes for them**

polymerization site «a». MAb II-4d, which reacts with fibrinogen, soluble fibrin and D-dimer, has been used for the design of three immunodiagnostic test-systems for quantification of these molecular markers of thrombophilia in human blood plasma as the tag-antibody.

By virtue of fibrin-specific mAb FnI-3C we discovered an neoantigenic determinant in fibrin molecule, which is exposed after the conversion of fibrinogen into fibrin. This determinant is located in Bβ126-135 and coincides with the epitope for mAb I-3C and with the site, which plays functional role in the fibrin protofibrils lateral association. Using this mAb the functional role of coiled-coil connector of fibrin molecule in the fibrin polymerization was showed for the first time. MAb I-3C reacts only with fibrin, does not react with fibrinogen and D-dimer and can be used in double-sandwich ELISA test-system for soluble fibrin quantification in human blood plasma as a «catch»-mAb for diagnostics of the threat of thrombus formation.

Finally, three mAbs to αC-connector of fibrinogen were produced. MAb II-2M of IgM class allowed us to confirm binding of αC-connector to fibrinopeptides B in fibrinogen and fibrin desA molecules. The presence of two polymerization sites in C-terminal parts of αC-connector was also shown using other mAbs I-5B and I-6B.

Thus monoclonal antibodies proved to be very useful for discovering unknown antigenic determinants and polymerization sites within the fibrin molecule; for the study of the three-dimensional structure of the fibrin molecule and for designing of test-systems to diagnose the threat of the intravascular thrombus formation.

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ВИКОРИСТАННЯ МОНОКЛОНАЛЬНИХ АНТИТІЛ ДЛЯ ВИЗНАЧЕННЯ ЛОКАЛІЗАЦІЇ АНТИГЕННИХ ДЕТЕРМІНАНТ І САЙТІВ ПОЛІМЕРИЗАЦІЇ ФІБРИНУ ВСЕРЕДИНІ МОЛЕКУЛ ФІБРИНОГЕНУ ТА ФІБРИНУ І ЇХ ЗАСТОСУВАННЯ У ТЕСТ-СИСТЕМАХ ДІАГНОСТИКИ ТРОМБОУТВОРЕННЯ

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Із використанням моноклональних антитіл було показано, що BβN-регіон молекули фібрину desA (Bβ1-53) містить сайт полімеризації, що включає пептидний зв'язок Bβ14-15, який бере участь у другій стадії полімеризації фібрину — латеральній асоціації протофібрил.

ИСПОЛЬЗОВАНИЕ МОНОКЛОНАЛЬНЫХ АНТИТЕЛ ДЛЯ ОПРЕДЕЛЕНИЯ ЛОКАЛИЗАЦИИ АНТИГЕННЫХ ДЕТЕРМИНАНТ И САЙТОВ ПОЛИМЕРИЗАЦИИ ФИБРИНА ВНУТРИ МОЛЕКУЛ ФИБРИНОГЕНА И ФИБРИНА И ИХ ПРИМЕНЕНИЕ В ТЕСТ-СИСТЕМАХ ДИАГНОСТИКИ ТРОМБООБРАЗОВАНИЯ

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С использованием моноклональных антител было показано, что BβN-регион молекулы фибрина desA (Bβ1-53) содержит сайт полимеризации, включающий пептидную связь Bβ14-15, которая принимает участие во второй стадии полимеризации фибрина —

У фрагменті Bβ15-53 також виявлено сайт, названий «С», який разом із сайтом «А» бере участь у першій стадії полімеризації — утворенні протофібрил. Створено модель первинної міжмолекулярної взаємодії фібрину. В N-кінцевій половині γ-ланцюга D-регіону фібрину із застосуванням моноклональних антитіл II-4d було виявлено сайт («с»), який бере участь у побудові протофібрил і, за нашим припущенням, є комплементарним сайту «С».

Знайдено дві неоантигенні детермінанти, одна з яких експонується в суперспіральному фрагменті фібрину Bβ126-135 в результаті відщеплення фібринопептидів А від фібриногену тромбіном. Зміна просторової структури молекули фібрину, що її виявлено в цьому сайті, є необхідною для здійснення подальшої латеральної асоціації протофібрил. Друга неоантигенна детермінанта формується у фрагменті Bβ134-190 D-димеру, який утворюється під час розщеплення фібрину, стабілізованого FXIIIa, плазміном. Проти першої детермінанти отримано фібринспецифічне моноклональне антитіло FnI-, а проти другої — D-димер-специфічне III-3b.

Одержано 3 моноклональних антитіла проти αC-регіону молекули фібрин (оген)у. За допомогою одного з них було експериментально показано, що αC-домен з'єднаний з фібринопептидом В у фібриногені та у фібрині desA. Цей домен відходить від остова молекули після відщеплення фібринопептидів В тромбіном. Два інших моноклональних антитіла специфічно інгібують полімеризацію фібрину блокуванням двох невідомих сайтів полімеризації в αC-регіоні.

На основі моноклональних антитіл FnI- і III-3b, використовуваних як «catch»-антитіла, і антитіл II-4d — як «tag»-антитіла розроблено тест-системи для кількісного визначення розчинного фібрину та D-димеру в плазмі крові людини. Ці тест-системи апробовано в клінічних випробуваннях в Україні. Показано, що для прогнозування післяопераційних тромботичних ускладнень, а також для контролю ефективності антитромботичної терапії є необхідним одночасне кількісне визначення розчинного фібрину та D-димеру до операції та в різні періоди після операції. Тільки в цьому разі можна отримати інформацію про стан балансу між системами зсідання крові та фібринолізу і визначити ступінь загрози тромбоутворення.

Ключові слова: фібриноген, фібрин, моноклональні антитіла, тромбоутворення, діагностичні тест-системи.

латеральной ассоциации протофибрилл. Во фрагменте Bβ15-53 также обнаружен сайт, названный «С», который вместе с сайтом «А» принимает участие в первой стадии полимеризации — образовании протофибрилл. Создана модель первичного межмолекулярного взаимодействия фибрина. В N-концевой половине γ-цепи D-региона фибрина с применением моноклональных антител II-4d был обнаружен сайт («с»), который принимает участие в построении протофибрилл и, по нашему предположению, комплементарен сайту «С».

Обнаружены две неоантигенные детерминанты, одна из которых экспонируется в суперспиральном фрагменте фибрина Bβ126-135 в результате отщепления фибринопептидов А от фибриногена тромбином. Изменение пространственной структуры молекулы фибрина, выявленное в этом сайте, необходимо для осуществления последующей латеральной ассоциации протофибрилл. Вторая неоантигенная детерминанта формируется во фрагменте Bβ134-190 D-димера, образующемся при расщеплении фибрина, стабилизированного FXIIIa, плазмином. Против первой детерминанты получено фибринспецифическое моноклональное антитело FnI-, а против второй — D-димер-специфическое III-3b.

Получены 3 моноклональных антитела против αC-региона молекулы фибрин(оген)а. С помощью одного из них было экспериментально показано, что αC-домен соединен с фибринопептидом В в фибриногене и в фибрине desA. Этот домен отходит от остова молекулы после отщепления фибринопептидов В тромбином. Два других моноклональных антитела специфически ингибируют полимеризацию фибрина путем блокировки двух неизвестных сайтов полимеризации в αC-регионе.

На основе моноклональных антител FnI- и III-3b, используемых в качестве «catch»-антител, и антител II-4d — в качестве «tag»-антител разработаны тест-системы для количественного определения растворимого фибрина и D-димера в плазме крови человека. Эти тест-системы апробированы в клинических испытаниях в Украине. Показано, что для прогнозирования послеоперационных тромботических осложнений, а также для контроля эффективности антитромботической терапии необходимо одновременное количественное определение растворимого фибрина и D-димера до операции и в разные периоды после операции. Только в этом случае можно получить информацию о состоянии баланса между системами свертывания крови и фибринолиза и определить степень угрозы тромбообразования.

Ключевые слова: фибриноген, фибрин, моноклональные антитела, тромбообразование, диагностические тест-системы.

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IMMUNOBIOLOGY OF DIPHTHERIA. RECENT APPROACHES FOR THE PREVENTION, DIAGNOSIS, AND TREATMENT OF DISEASE

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Diphtheria is a highly contagious life-threatening disease caused by the toxigenic strains of *Corynebacterium diphtheria*, which are transformed by a bacteriophage carrying the toxin gene. Diphtheria causative agent and its major virulence factor diphtheria toxin are well studied, but outbreaks of disease still occur worldwide. Rapid development of new methods in immunology and molecular biology is currently leading to improvement of prophylaxis, diagnosis and treatment of diphtheria. This review highlights the microbiological, epidemiological and immunological aspects of diphtheria infection, role of diphtheria toxin and others virulence factors in diphtheria pathogenesis and role of humoral anti-toxic immunity in the protection against disease. Perspectives in development of new diagnostic tests, anti-diphtheria vaccines, immunobiological preparations and antidotes for prevention of diphtheria infection, and other anti-diphteria means was also discussed.

Key words: diphtheria, diphtheria toxin, immunity, diagnostic tests, vaccines, antidotes, recombinant proteins.

Diphtheria is an acute infectious disease caused by the bacterium *Corynebacterium diphtheriae* (also known as Klebs-Löffler bacillus) [1]. Typically, diphtheria has respiratory or cutaneous localization. Respiratory diphtheria has various forms, usually restricted to upper respiratory tract: nasal, pharyngeal, tonsillar and laryngeal. As rule, main symptoms of respiratory diphtheria are sore throat, low fever, and an adherent membrane at the site of bacterial colonization [2]. Milder forms of diphtheria are often restricted to the skin [3].

Long time diphtheria was considered as well-controlled vaccine-preventable disease because it has largely been eradicated in all industrialized countries presumably through broad vaccination [4–6]. However, a diphtheria epidemic at the former Soviet Union territory at 1990s has again attracted the attention to incomplete understanding of the epidemiology, microbiology and especially immunobiology of this infection [7–12].

Today cases of diphtheria are still occur in Ukraine, Russia, and Latvia and also it is endemic in India, Indonesia, Nepal, Angola and Brazil, but only sporadic cases are reported in developed countries [11, 13–16]. However, the majority of the adult populations in Europe, Australia and the United States have no immune protection against this infection [13, 17]. This issue draws renewed attention to the immunology of this infection, because lowered immunity levels within population can cause outbreaks of diphtheria.

Bacterial pathogenesis

Biological properties of *C. diphtheriae*. *C. diphtheriae* is an aerobic nonmotile, rod-shaped gram-positive bacillus, which can form metachromatic granules at the ends of the rod. Bacterial cells form irregular V-shaped aggregates resembling Chinese letters [1]. Species *C. diphtheriae* has three biotypes: *gravis*, *intermedius*, and *mitis*, which are differ by the colony morphology and growth characteristics [18]. Genomic sequence of *C. diphtheriae* has been recently characterized [19], but molecular basis for differences in *C. diphtheriae* biotypes is not well defined and requires further investigation [20, 21]. The most severe diseases are often associated with the *gravis* biotype, but

every strain has ability to produce toxin. Consequently, all isolated strains of *C. diphtheriae* should be tested in the laboratory for toxigenicity [22].

Besides *C. diphtheriae* there are two other species *Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis*, which can optionally produce diphtheria toxin and hence can cause respiratory illness resembling classical diphtheria [23–25]. It is worth noting that in recent years some severe infections caused by *C. ulcerans* have been recorded [26, 27]. Other well-known human pathogen *C. pseudodiphtheriticum* causes respiratory disease and is associated with high mortality in compromised hosts [28], however it is little known about the virulence factors and pathogenesis of such infections. Some other species of *Corynebacterium* are part of human normal flora, which able to find niches in every suitable anatomic location of the host [29].

Most the clinical manifestationsof diphtheria result from the action of an exotoxin produced by pathogen. Consequently, diphtheria toxin (DT) produced by toxigenic strains of *C. diphtheriae* is considered as the main pathogenic factor of infection. Toxigenicity of *C. diphtheriae* is controlled by bacteriophage conversion [30–32]. Thus toxin production occurs only when the bacterium is infected by lysogenic corynephage carrying the *tox* gene encoding DT.

Production of DT by toxinogenic *C. diphtheriae* is strictly repressed by high concentrations of iron ions from the extracellular environment. The main source of the iron at site of bacterial colonization is the red blood cells, which appear from the bleed via damaged mucous epithelium [33]. The chromosomally encoded diphtheria toxin repressor DtxR depends of iron ions as a co-repressor factor [34]. The iron bound form of DtxR binds to DNA sequence and prevents initiation of transcription of bacteriophagal *tox* gene. Hence, DtxR can be considered as iron-dependent transcriptional repressor [35]. In bacterial cell, DtxR regulates several chromosomally encoded genes, which encode products involved in iron utilization and acquisition [36, 37]. Some mutant forms of DtxR or iron-regulated promoters could constitutively repress the expression of diphtheria toxin gene, and phenotypically nontoxigenic strains may represent a potential reservoir for the emergence of toxigenic strains [38–40].

Strains of *C. diphtheriae* that do not produce diphtheria toxin are still frequently considered as non-virulent. Nevertheless, the association of nontoxigenic strains with localized disease is well known. There is an option that nontoxigenic strains may be responsible for pharyngitis and should be treated [22, 41, 42]. However, additional studies are still required to obtain complete information about the pathogenicity or co-pathogenicity of nontoxigenic *C. diphtheriae* associated with cases of infection in the respiratory tract [43].

The introduction of a toxigenic strain of *C. diphtheriae* into a community may initiate an outbreak of diphtheria by bacterial spreading or by transfer of the bacteriophage to nontoxigenic strains carried in the respiratory tracts of susceptible human subjects. Both toxigenic and nontoxigenic strains of *C. diphtheriae* could be isolated during outbreak of infection, but the epidemiological role of nontoxigenic strains is under the question [44, 45].

Only toxigenic strains can cause respiratory diphtheria, conversely nontoxigenic strains can live in the organism without any clinical manifestation or cause some other pathologic states.

Recently, it was shown that nontoxigenic strains are associated with cases of invasive infection, particularly with endocarditis [46–48]. These microorganisms also can be associated with other invasive diseases, such as septic arthritis and osteomyelitis [49], or catheter-related infection [50].

The systemic diseases caused by *C. diphtheriae* often related to invasive clones. Invasive diseases add new aspects to the infectious processes caused by *C. diphtheriae*. Entry of *C. diphtheriae* by invasive processes can be caused by percutaneous trauma, skin and throat colonization. Unlike classical diphtheria, invasive disease caused by *C. diphtheriae* affects both vaccinated and non-vaccinated persons, and mostly induced by nontoxigenic isolates.

The patterns of adherence to HEp-2 cells (epidermoid carcinoma tissue from the larynx) of *C. diphtheriae* strains can be used to predict their invasive character [48, 51]. Invasive microorganisms yielded simultaneous expression of localized adherence-like and aggregative-like adherence patterns to HEp-2 cells. Microbial adhesive properties may contribute to the spread and outcome of invasive processes.

Diphtheria toxin. Since the discovery of diphtheria toxin by Roux and Yersin in 1888 [52] it became one of the most extensively studied bacterial toxins. The minimal lethal dose of diphtheria toxin for humans and animals is below 0.1 mkg per kg of body weight [53]. The delivery of a single molecule of diph-

theria toxin to the cell is sufficient to kill a eukaryotic cell [54].

Diphtheria toxin is an A-B type toxin consisting of two fragments: A (active) and B (binding) (Fig. 1). This protein consists of three domains: catalytic C-domain, transmembrane T-domain and receptor-binding R-domain. C-domain encompasses the fragment A (SubA — subunit A), T-domain and R-domain together constitute the fragment B (SubB — subunit B) [55].

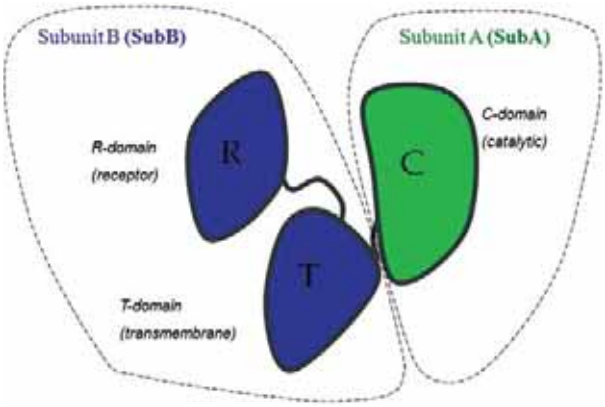

Fig. 1. **Schematic structure of diphtheria toxin (DT)**

Receptor for DT (Fig. 2) is well characterized [56, 57]. Membrane-anchored precursor of heparin-binding epidermal growth factor-like growth factor (pro-HB-EGF) binds with toxin with high affinity (Kd of approximately 10^{-8} –10^{-9} M) [58]. DT is only one known natural ligand for pro-HB-EGF, which causes its internalization.

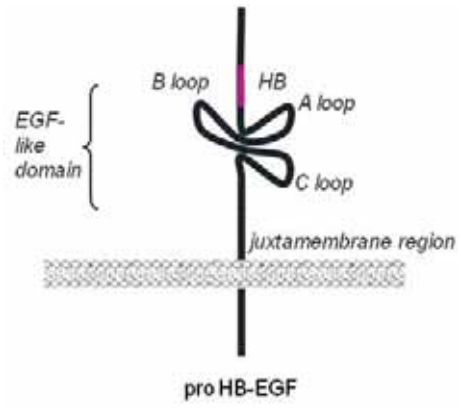

Fig. 2. **Schematic structure of the DT receptor — heparin-binding EGF-like growth factor (HB-EGF) exposed on the cell surface in the form of profactor. (The Subunit B of DT binds to the EGF-like domain of HB-EGF)**

HB-EGF is a member of the EGF family growth factors, which has high affinity for heparin and heparan sulfates [59]. Pro-HB-EGF is synthesized as type I transmembrane protein, which after processing by metalloproteases like ADAMs turns into soluble form (sHB-EGF). sHB-EGF acts as ligand for the EGF receptors of I and IV type, thus it is considered as a potent mitogen and chemoattractant for different cell types, including malignant cells.

The first step in intoxication of eukaryotic cells by diphtheria toxin is binding of the toxin to a specific cellular receptor pro-HB-EGF. The fragment B of DT is responsible for interaction with receptor on the cell surface and translocation of the fragment A across endosomal membrane into the cell cytosole. Two domains of fragment B have different functions. R-domain mediates binding of DT to its surface receptor, which promotes endocytosis of the toxin-receptor complex. T-domain facilitates C-domain translocation across lipid bilayer.

Upon endosome formation, endosomal low pH induces conformational changes that result in diphtheria toxin T-domain interaction with the endosomal membrane [60].

Thereafter T-domain mediates translocation of C-domain into the cell cytosol. Mechanism of C-domain translocation remains unclear, but obvious that it depends on conformational switching of T-domain and its affinity to proteins in molten globule state.

After C-domain translocation across endosomal membrane, it restores the ability to inactivate eukaryotic translation elongation factor 2 (eEF2). Subunit A possesses ADP-rybosyl transferase activity and specifically inactivates eEF2. Accumulation of large number of inactivated eEF2 leads to inhibition of cellular protein biosynthesis and cell death [61].

The target of ADP-dribosylation by diphtheria toxin is unusual amino acid residue in eEF-2-diphthamide (a posttranslationally modified histidine). The diphthamide residue is unique to EF-2 from eukaryotes and Archea [62]. Synthesis of diphthamide is a complex process that requires the participation of several different proteins [63]. It has been suggested that ADP ribosylation of diphthamide in EF-2 may occur as a regulatory event in normal cellular physiology, but evidence for the precise physiological role of diphthamide in eukaryotes remains unknown [64]. Mutant cell lines that cannot produce diphthamide remain viable and are resistant to diphtheria toxin [65].

There are several important questions regarding DT functions remain to be investigated, like mechanism of translocation of the A fragment across endosomal membranes, mechanism of rodent toxin-resistance, the physiologic role of diphthamide residue of EF-2 in eukaryotic cells, as well as mechanism of immune recognition and protection mediated by toxin-specific antibodies etc.

Diphtheria toxin is responsible for the local cell damage at site of bacterial colonization as well as for distant toxic effect on peripheral nervous system, kidneys and heart. Apparently DT also helps bacteria to evade immune defense mechanisms and to escape from phagocytosis. Thus, DT and probably other surface structures of *C. diphtheriae* toxigenic strains show apoptogenic effect on mice peritoneal macrophages *in vitro*[66]. Small amounts of toxin can impair protein synthesis in both polymorphonuclear leukocytes and mononuclear cells from humans and guinea pigs [67]. DT could penetrate into phagocytes and B-cells specific to DT and kill these cells even if they derived from toxin-resistant animals [68]. This observation confirms that DT is potentially able to inhibit self-directed antibody response and phagocytosis and in this way escape from host defense mechanisms.

Additional bacterial virulence factors. Despite that the role of DT in bacterial virulence is well established, there are little known about other virulence factors of *C. diphtheriae*. These factors could be crucial for colonization of the host and recognition of corresponding host receptors since colonization is an essential step in pathogenesis. However, host cell receptors and invasion-associated proteins of the pathogen remains unknown.

On the HEp-2 cell system was shown distinct patterns of bacterium adherence: an aggregative, a localized and a diffuse [48, 51], which confirm an existence of several adhesion factors and different receptors on the host cell surface. Some bacterial adhesion factors have been recently characterized on the molecular level. Certain *C. diphtheriae* strains able to express three types of pili (SpaA, SpaB and SpaC) on its surface [69, 70], which are sufficient for adhesion to pharynx cells. There some additional proteins besides pili proteins involved in adhesion to larynx, pharynx and lung epithelial cells. For instance, *C. diphtheriae* invasion-associated protein (DIP1281) involved in cell surface organization, adhesion and internalization in epithelial cells [71]. In addition, the disruption of the *C. diphtheriae* DIP1621 gene leads to decreased adherence to epithelial cells [72]. DIP0733 (67-72p) may be directly implicated in bacterial invasion and apoptosis of epithelial cells in the early stages of diphtheria and *C. diphtheriae* invasive infection [73]. Non-fimbrial surface protein 67-72p also involved in adhesion to human erythrocytes. Iron supply has effect on binding properties of the microorganisms to erythrocytes as well as HEp-2 cells [74].

Number of studies suggesting the multifactorial mechanism of adhesion [75]. In addition, biofilm formation and fibrin deposition may contribute to the persistence of *C. diphtheriae* at the infected site [50].

Intracellular survivelance strategy. *C. diphtheriae* generally considered an extracellular colonizer. However, some strains of *C. diphtheriae* possess the ability to enter into cells and to survive within cultured cells [51, 76]. *C. diphtheriae* strains can adhere to epithelial cells and erythrocytes and has ability to survive within these cells. Probably, *C. diphtheriae* strains might use epithelial cells as an environmental niche supplying protection against antibodies and macrophages [71]. Some *C. diphtheriae* strains even without the *tox* gene exhibit strategies to survive within macrophages and to exert apoptosis and necrosis in human phagocytic cells [77]. Invasion of these cells is an active process; tetracycline-treated *C. diphtheriae* was still able to attach to host cells, but lost its ability to invade the cytoplasm [78]. As rule, the interaction between bacteria and macrophage determines the outcome of most infectious diseases.

The ability of diphtheria infectious agent to cause macrophage apoptosis is one of the mechanisms of realization of its pathogenic properties determined by the effect of diphtheria exotoxin, as well as its surface structures and pathogenicity enzymes. The presence of the *tox* gene influences the susceptibility of *C. diphtheriae* to human macrophages and the outcome of non-opsonic phagocytosis [77]. Analyses of molecular mechanisms of non-opsonic phagocytosis should lead to new approaches for the prevention of diphtheria and systemic *C. diphtheriae* infections. Homologous *C. diphtheriae* tox^+ and tox^- strains can survive within U-937 human macrophages but viable intracellular bacteria can be detected after 24 hr only for the tox^- strain.

Pseudomembrane formation and coagulase-like activity. The characteristic feature of disease is a pseudomembrane formation that usually covers the posterior pharynx and

tonsils, which may also extend to the larynx and lower respiratory tract [2, 79]. Pseudomembrane of respiratory diphtheria is composed of fibrin matrix with incorporation of bacteria, necrotic epithelial and inflammatory cells, which adheres tightly to the underlying tissue [2]. The severity of the disease usually related to the extent of the local infection, although the potential role of these pseudomembranes in the maintenance of viable *C. diphtheriae* is still uncharacterized.

Although fibrin pseudomembrane is a characteristic feature of diphtheria, there is little known about the fibrinogen-binding properties and fibrin clot formation activity of *C. diphtheriae* strains and the role of the DT in these processes. The production of fibrinous exudates may play an important role in determining of the pseudomembrane formation. Diphtheria toxin generally considered as the major factor responsible for local cellular destruction and production of fibrinous exudates, suggesting that the presence of bacteriophages carrying the diphtheria toxin gene (*tox*) is essential for pseudomembrane formation. Recently reported property of *C. diphtheriae* iscoagulase-like activity. The capacity to bind to fibrinogen and to convert fibrinogen to fibrin may play a role in pseudomembrane formation and act as virulence determinants for both nontoxigenic and toxigenic strains [43].

Consequently, production of DT is important for the epithelial cell damage and production of fibrinous exudates, while coagulase-like activity of *C. diphtheriae* may be important forfibrin polymerization. That is why we could consider DT as molecular instrument used by *C. diphtheriae* at the site of colonization for the partial epithelial cell damage in order to get small portion of fibrinogen from the blood for the fibrin formation. After short bleeding, the production of DT is inhibiting by iron ions from incoming hemoglobin. DT also can get to the blood circulation via damaged epithelia and thus cause severe systemic toxic effects. Diphtheria toxin exerts its effects on distant tissues and organs, especially the heart (causing myocarditis), and the peripheral and cranial nerves (causing weakness progressing to paralysis), if absorbed from the site of infection.

Assessment of anti-diphtheria protection

Serologic methods of diphtheria diagnosis based on the detection of diphtheria toxin or on increased level of antitoxic antibodies. Therefore, measurement of antitoxin level in diphtheria patients could provide important clinical information about course of infection.

In addition, determination of anti-toxin antibodies is essential for characterization of the immune status of population, and evaluation of the immunogenicity of diphtheria vaccines in clinical trials, as well as for monitoring long-term immunity and thus provides recommendations for vaccination policy. Data obtained from serological studies serve as an important guide in choosing of local strategy of vaccination. Detecting the existence of a cohort of susceptible subjects can predict the risks for disease outbreaks. Therefore, it is of critical importance to have methods for assessment of anti-diphtheria immunity that are accurate, reproducible, specific, and sensitive.

Most symptoms of diphtheria are resulted from the diphtheria toxin action; therefore, protection against disease depends on antibody level against the toxin (antitoxin). The assessment of the anti-diphtheria protection in healthy population is common for a surveillance system within any National Program of Immunization. According to the Order № 545 of Ministry of Health of Ukraine from 24.11.2003 «About Ukrainian population immunity against diphtheria and tetanus», clinicists also need «to provide annual studies of population immunity to diphtheria and tetanus among healthy population (1.2.)»

Antitoxic antibodies probably play a main role in the immunity against diphtheria. Serum titers of antitoxin usually are expressed in International Units per milliliter (IU/ml) according to the diphtheria antitoxin standard. The cut-off of protective serum level of antitoxin is 0.01 IU per ml. (but it also depends on the method of titer determination). As believed, the powerful anti-toxin immunity (>1.0 IU/ml) can completely protects the body from infection caused by toxigenic strains. Although, the very little is known about protection associated with nontoxigenic strains.

Classical serological tests tend to underestimate low concentrations of diphtheria antibody. That is why antitoxin level under 0.1 IU per ml could not be defined precisely in many laboratories where hemagglutination test is used for this purposes. In clinical interpretation of results, antibody titers classified into one of the following categories: insufficient protection (<0.1 IU/ml), satisfactory protection (0.1–1.0 IU/ml) and high levels of protection (>1.0 IU/ml) [44]. However, with more reliable techniques it is possible to define an

additional categories like short-term protection (0.01–0.1IU/ml) and no protection (<0.01 IU/ml).

Numerous *in vivo* and *in vitro* tests for the measuring of diphtheria antitoxin levels in serum have been standardized and implemented for laboratory practice. Among the *in vivo* protocols are the Schick test in humans and the classical toxin neutralization (TN) assay in rabbits or guinea pigs. There is also the *in vitro* toxin neutralization test in microcell culture plates using highly sensitive Vero (green monkey renal epithelium) cell line [80]. Several *in vitro* serologic techniques for diphtheria antitoxin determination are described [81].

Toxin neutralization tests. DT is a toxic agent that can kill eukaryotic cells and can cause systemic reaction in sensitive organisms. Protective antibodies can block specific binding of DT to cell receptor and therefore to protect the cell and the body from toxic action of DT. Existing toxin neutralization tests exploit *in vivo* or *in vivo* models oftoxicity for measuring the level of antitoxin by dose-depended neutralization effect.

DT can cause *in vivo* inflammatory response when injected in small doses intracutaneously into the skin of humans or sensitive animals. The ability of specific antibodies to prevent this reaction can be utilized to determine the activity of antitoxin. Thus, the *in vivo* neutralization tests show the functional capacity of antibody to neutralize toxin in live organisms.

One of the first methods to estimate immunity against diphtheria in humans was intradermal Schick test with active diphtheria toxin [82]. Bŭla Schick designed this test in 1913 as an approach to determine susceptibility to diphtheria in children. This method allows controlling the population immunity against diphtheria. At that point, immunization was available for those who had never been naturally immunized by exposing to live diphtheria bacilli. This test eventually led to the eradication of the childhood disease, made Bŭla Schick world famous [83].

The Schick test involves injecting a very small amounts (0.1 ml of diluted 1/50 MLD for the guinea pigs) of the toxin into the skin of the forearm and evaluating the reaction at the injection site after 48 hours [84]. The result of positive test manifested in inflammatory reaction indicates susceptibility to diphtheria, whereas result of negative test without any reaction indicates immunity (antibody neutralizes toxin). A control injection with inactivated toxin had to be performed to exclude allergic reactions to toxin. Schick test results usually correlate well with serum antitoxin levels. The average antitoxin level up to 0.1–0.3 IU/mL is corresponded to the negative Schick test reaction when antibodies can completely neutralize injected toxin. However this test is no longer used in healthcare due to safety requirements, painful effect when results are positive, time-consuming, need for two visits, occurrence of pseudo negative reaction, etc. Currently, passive hemagglutination reaction with red blood cells is commonly used for this purpose.

There are several biological tests on sensitive animals are also available to quantify the level of antitoxin. The *in vivo* neutralization tests can be performed on rabbits (Jensen, 1933) or guinea-pigs (Glenny & Llewellyn-Jones, 1931). Different dilutions of serum mixed with fixed amounts of diphtheria toxin can be injected into the depilated skin of the animal, and the antitoxin concentration could be estimated based on the presence or absence of an inflammatory reaction. The *in vivo* toxin neutralization test using guinea pigs or rabbits is referred as the «gold standard» method for determining protective levels of antitoxin in serum. The toxin neutralization assay has been recognized as an accurate and sensitive test able to detect antitoxin levels as low as 0.001 IU/mL [85]. However, this test requires large numbers of animals, relatively large volumes of serum as well as specialized facilities and personnel trained to work with animals. Therefore, this test is highly expensive and time-consuming, thus it is not convenient for practical use in serological diagnosis or epidemiological monitoring.

Consequently, *in vitro* methods as alternative to *in vivo* approaches can reduce time, costs and improve animal welfare. *In vitro* tests with cultured cells (neutralization test on microcell culture) have been developed as “humane” alternatives to the *in vivo* test for detection of diphtheria antitoxin [86]. The ability of diphtheria toxin to cause cell death in cultured mammalian cells used to determine diphtheria toxin or antitoxin amounts. Thisneutralization test based on the observation that the presence of antitoxin in serum samples can promote survival of sensitive mammalian cells in culture treated with DT in dose-dependent manner.

Vero cells [87] commonly used in neutralization tests *in vitro* are most sensitive to diphtheria toxin cells since they have largest numbers of receptors on their surface [88].

Results of *in vitro* neutralization test read as a change in color of the medium (from red to yellow) in the cell-culture plate wells after 3–4 days of incubation. Only alive cells can change the medium color due to the metabolic formation of acid, which changes the pH of medium. Treated with DT cells retain their ability to grow when serum samples contains antitoxin in sufficient amount [80].

The *in vitro* neutralization test in microcell culture is highly sensitive (minimum detectable level is 0.005 IU/mL) and provides comparable results to *in vivo* neutralization test on guinea pig and rabbit skin [89–91]. Thus, the Vero cell toxin neutralization assay is recommended by World Health Organization and European Pharmacopeia as *in vitro* alternative method for guinea pig assay for potency testing of vaccines [92, 93].

Alternatively, cell culture test without native DT use was proposed. In these test native DT replaced with recombinant fusion protein consisting of B-subunit of DT and enhanced green fluorescent protein (EGFP-SubB) (Fig. 3). This protein was able to bind to DT receptor on Vero cells surface, but had no toxicity due to absence of C-domain [94]. This method based on the ability of anti-toxin antibodies to block the binding of fluorescently-labeled recombinant B subunit of DT to the cell surface receptor of Vero cells [95]. We called this method Vero-cell based toxin-binding inhibition test (Vero-ToBI). Proposed *in vitro* method for quantitative evaluation of protective antibodies in sera is significantly rapid than existing tests and not require native DT.

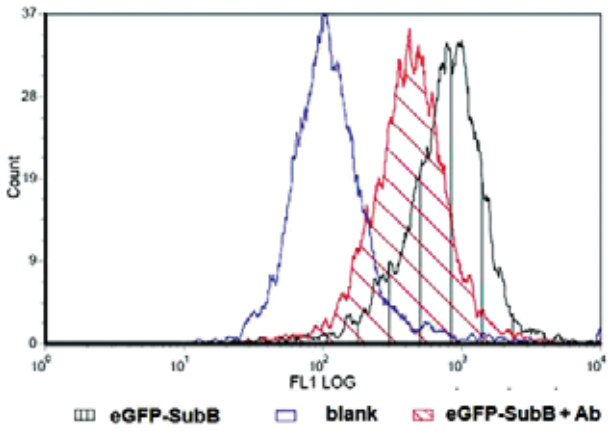

Fig. 3. **The Vero-cell based toxin-binding inhibition test. This method is based on the ability of anti-toxin antibodies to block the binding of fluorescently-labeled recombinant B subunit of DT to the cell surface receptor. This process could be measured by flow-cytometry**

Most important practical issues with all cell-culture tests are the relatively high complexity and time-consuming of the procedure, skilled staff and special laboratory equipment requirements. Therefore, a number of diagnostic laboratories may prefer to use more simplified format of diagnostic kits like serological assays.

Serological tests. For diagnostic and monitoring purposes, serological test can offer significant advantages in terms of cost, speed, ease of use and adaptability to automation. There are a number of serological methods for the estimation of antitoxin level available, like the passive hemagglutination assay [96] and the latex agglutination test [97], toxoid or toxin based ELISA, the double-antigen ELISA [98] and the double-antigen dissociation-enhanced lanthanide fluorescence immunoassay [99], as well as the toxin binding inhibition assay [100] and multiplex assay [101] etc.

Currently, passive hemagglutination reaction (PHA) with red blood cells is still the most frequently used method in many laboratories for the detection of anti-toxin antibodies. The PHA test use coated with diphtheria toxoid sheep red blood cells for agglutination by diphtheria antitoxin [96, 102, 103]. Overall it is relatively simple and inexpensive method, but there is poor correlation of PHA with contemporary toxin neutralization tests, which considered as standard reference methods. In addition, PHA test tends to underestimate low concentrations of antitoxin [104], lacks sensitivity and obviously needs improvement or replacement [81, 105]. Therefore, new assays for the detection of diphtheria antitoxin levels in the population extremely desirable.

An enzyme-linked immunosorbent assay can make the good alternative to PHA for the detection of anti-toxin antibodies. The indirect ELISA which is the simplest variant of this assay involves the estimation of antitoxin bounded to diphtheria toxin (or toxoid) adsorbed on ELISA plates [106]. The almost exact correlations between both the Toxoid-ELISA and the Toxin–ELISA were indicated [81]. Indirect ELISA tests in addition have the ability to measure class-specific antibodies such as IgG, IgM or IgA.

Results of the ELISA have high reliability and reproducibility. When the antibody titer is >0.1 IU/mL results of ELISA have good correlation with results of the neutralization tests in guinea-pigs [107] or in tissue culture[108], however there is poorer correlation with results of the neutralization test when the antibody titer is lower 0.1 IU/mL.

Better correlation can be achieved with modified ELISA tests [98, 100, 107] like the toxin binding inhibition test (ToBI-test) and the double-antigen ELISA, however potential drawback of these tests is inability to measure class-specific antibodies.

In the double antigen format of serologic tests one arm of the antibody binds to antigen immobilized on the plate and the other arm binds to labeled antigen providing enzymatic (DAE — double-antigen ELISA) [98] or fluorescent signal (DELFIA — dissociation-enhanced lanthanide fluorescence immunoassay) [99]. These assays showed a good correlation with established toxin neutralization assays. In addition, the double antigen system is indifferent for origin of serum permitting the use of calibration standard serum of animal origin to measure antitoxin levels in humans in international units (IU/ml). The detection limit for DELFIA format with Eu^{3+}-labeled toxoids corresponded to 0.0003 IU/ml. This fast with a high capacity assays can be alternative to above mentioned methods in serological surveillance studies [99].

High sensitivity and specificity together with the highest correlation with the reference test has ToBI–ELISA. The toxin binding inhibition test (ToBI-test) based on inhibition of the binding of toxin to an antitoxin-coated immunoassay ELISA plate by free antitoxic antibodies[100]. Consequently, the ToBI-test resembles classical sandwich-ELISA combined with competition assay. Antitoxin titers as low as 0.002 IU/ml were detectable by the ToBI-test, it is far below the level considered to be protective for human [100]. The ToBI-test shows good correlation (r = 0.91–0.93) with the *in vitro* neutralization test on Vero cells [100]. From samples with a titer below 0.1 IU/ ml, as estimated by the reference test, 96% were correctly identified by the ToBI–ELISA [81]. Antibody affinity thought to be a key factor to influence the resulting relative antibody titer in ToBI-test [109].

Apparently, determination of antibody titer against the whole toxin molecule cannot provide information about the precise contents of protective antibodies. Protective properties are inherent mainly to the antibodies against B-subunit of the toxin, because only these antibodies can inhibit the toxin binding to the receptor. Antitoxic antibodies to A-subunit of DT often predominate over antibodies to B-subunit in children with diphtheria (as opposed to carriers and vaccinated children) (Fig. 4) [110]. Therefore, recombinant A- and B-subunits of DT [111] was proposed to use in ELISA and flow chromatography test-systems for diphtheria diagnosis and for monitoring of vaccine efficiency. Differential assessment of antibodies to distinct DT fragments with recombinant analogues of A and B subunits can be used for a rough evaluation of protective anti-diphtheria antibodies. In addition, the information regarding level of antibodies to A-fragment of DT provides indirect information about the contact of the person with DT during naturally occurred immunization process.

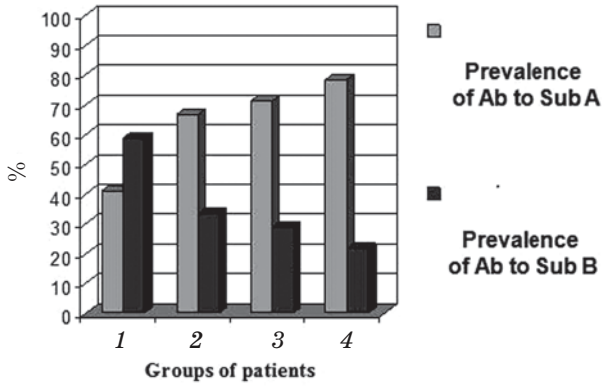

Fig. 4. **The specificity of serum antibodies to separated subunits of diphtheria toxin.**
1 — patients with diphtheria;
2 — carriers of toxigenic strains of *C. diphtheriae*;
3 — carriers of non-toxigenic strains of *C. diphtheriae*;
4 — healthy volunters immunized with DTP-vaccine

Recombinant soluble form of DT receptor sHB-EGF was proposed to use instead capture antibodies in sandwich ELISA for functionally active DT detection. Affinity constant for interaction of recombinant sHB-EGF with DT was similar to the affinity of natural sHB-EGF with DT. The developed sandwich ELISA allowed detection DT with sensitivity up to 1.9 ng/ml [112]. Another test-system for the detection of protective antibodies against DT was based on the competitive ligand-receptor enzyme immunoassay. Recombinant DT receptor sHB-EGF as the bottom layer (bait) and the enzyme-labeled toxin B-subunit as the second layer (prey) allowed identifying anti-toxin antibodies with protective properties (able to prevent the toxin-receptor interaction) (Fig. 5).

Developed methods for evaluation of protective anti-diphtheria immunity can be applied in clinics for monitoring the effectiveness of vaccination within the healthy population, as well as in search for new means of anti-diphtheria immunotherapy and immunoprophylaxis. (Fig. 6)

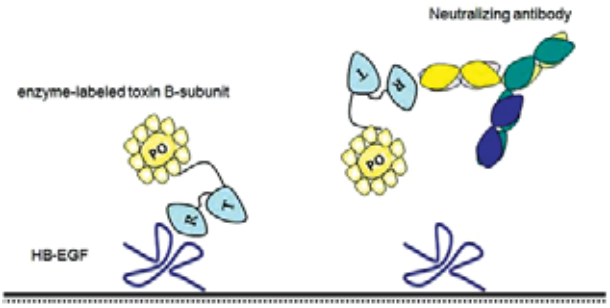

Fig. 5. **The competitive ligand-receptor enzyme immunoassay for the detection of protective antibodies against DT.** Application of recombinant DT receptor (HB-EGF) as the bottom layer (bait) and the enzyme-labeled toxin B-subunit as the second layer (prey) allows to identify anti-toxin antibodies with protective properties able to prevent the toxin-receptor interaction

Fig. 6. **Kit for the detection of protective antibodies against DT based on the competitive ligand-receptor enzyme immunoassay**

Immunity to diphtheria

Diphtheria toxin produced by *C. diphtheriae* during the disease or the carrier state has ability to induce production of naturally acquired antibodies against the toxin (antitoxin). Artificial immunity to diphtheria can be stimulated with diphtheria toxoid immunization. Antitoxin can pass through the placenta providing passive immunity to the infant during the first months of life. Patients can acquire passive immunity to diphtheria by injection of equine antitoxin in course of the disease therapy.

As supposed, the primary role in the protection against diphtheria belongs to the antibodies of IgG class, but protection potential of IgA and IgM antibodies is remains underestimated. As mentioned earlier, antibodies to B-fragment of DT are more protective than antibodies to A-fragment.

Recovery from diphtheria is also associated with activity of phagocytes at site of infection. However, there is little known about cell-mediated immune responses to toxin or toxoid and other antigenic substances of *C. diphtheriae*.

Passive immunity to diphtheria. Passive immunity to diphtheria can occur naturally when maternal antibodies are transferred to the fetus through the placenta. Thus, most infants have protective antitoxin level acquired passively from their mothers [113]. However, the half-life of passively acquired antitoxin by newborns is about 30 days [114], thus level of these antibodies significantly decreases between 6 and 12 months. Mothers and their infants have highest diphtheria antitoxin titers (above 0.1 IU/ml) in areas with normal circulation of toxigenic *C. diphtheriae* in population [115].

High titers of maternal antibodies can interfere with serologic response of infants to diphtheria vaccination. The modifying effect of passively-acquired maternal antibodies in young infants is strongest under the age of 4 weeks [116]. High titers of passively transferred antibodies may temporarily interfere with active immunization of infants [117, 118]. Maternal transferredantibodies may suppress responses to the first or second vaccination [119]. Thus in the countries where circulation of toxigenic *C. diphtheriae* is common the early immunization of infant is not so effective due to the presence of high level of maternal antitoxin. At the other hand, early immunization of these infants can deplete their passive immunity due to the absorbance of maternal antibodies by injected toxoid.

Equine diphtheria antitoxin and other antidotes. Passive immunity to diphtheria can be also induced artificially, when high levels of horse antibodies (DAT — diphtheria antitoxin) specific for toxin are transferred to non-immune individuals in order to prevent or cure disease [120]. DAT neutralizes circulating toxin and can prevent progression of the disease. However, DAT could not directly kill live microorganism colonizing mucous epithelia. Therefore, additional treatment with antibiotics required.

This antitoxin was first used in 1890s for prevention of the disease [121–123]; however, modern application of DAT involves only the diphtheria treatment, but not its prophylaxis [124]. Patients with diphtheria suspected have to be given antitoxin and antibiotics in adequate dosage and placed into isolation. The treatment with DAT has serious drawbacks resulted in serum sickness, an immune complex disease, thus a better treatment for diphtheria patients remains desirable [125].

Ways to reduce immunogenicity and allergenicity of therapeutic equine antibodies are to decrease their molecular mass, to modify their Fc fragment and/or to humanize them. Therefore, monoclonal human antibodies, humanized rabbit or mouse antibodies, recombinant single chain fragment variable (scFv) antibodies with molecular mass of 25–35 kDa and camel nanobodies with molecular mass of 15 kDa are now considered as perspective toxin-neutralizing agents [126].

Antibody gene cloning coupled with phage display technique seems to be a relatively fresh solution for the issue of developing better therapeutic means. Generated antitoxic murine and human scFv antibodies have high affinity constants to B-subunit of DT (up to 10^9 M^{-1}) and could neutralize toxin binding to its receptor [127, 128]. Therefore, recombinant scFv antibodies against DT can be utilized for developing new therapeutic reagents.

Another concept of diphtheria treatment is based on preventing diphtheria toxin binding to its cellular receptor pro-HB-EGF by the soluble form of HB-EGF. In order to minimize its side effects sHB-EGF lacking grows-factor activity proposed [129].

Natural acquired active immunity to diphtheria occurs when a person is exposed to a live pathogen, and develops a primary immune response, which leads to immunological memory. When toxigenic *C. diphtheriae* commonly circulated in population natural immunity to diphtheria can be acquired with unapparent infection. Subsequently immunity rise rapidly in early childhood reflecting increasing exposure to diphtheria microorganisms. In the pre-vaccination era diphtheria was primarily an infection of children. At some developing countries at the age of 10–15 years almost all individuals had natural acquired immunity to diphtheria [130–132]. This pattern was observed in Europe and the United States in pre-vaccination era [133, 134] and in developing countries until nowadays.

Residual coetaneous diphtheria is considered as an ongoing source of natural immunity, but insufficient data are available regarding the current prevalence of skin infections [135, 136]. Furthermore, exposure to live *C. diphtheriae* can determine natural boosting of diphtheria immunity after vaccination. The low level of antitoxin among adults in developed countries may result from reduced exposure to live toxigenic microorganisms and thus reduced opportunity to acquire naturally immunity [15] (Schou et al., 1987; Simonsen et al., 1987; Simonsen, 1989). That is why the schoolchildren in developed countries sometime have low titers of antitoxin.. Thus, many authors emphasize the urgency of general revaccination against diphtheria of schoolchildren and adults [137–142].

Vaccines. Invention of toxoid in 1923 by Gaston Ramon provided safe and effective means for vaccination [143]. Formaldehyde treatment of DT eliminates its enzymatic activity and ability to bind to cell receptor, but retains its antigenic properties. In addition, formaldehyde treatment enhances immunogenicity of toxoid while preserving its structural integrity and ability to induce highly active toxin-neutralizing antibodies [144, 145]. Such treatment converts toxic DT to harmless toxoid, which is widely used for immunization against diphtheria. Diphtheria toxoid is still the basis of current anti-diphtheria vaccines. Diphtheria toxoid in vaccines presented most commonly alone or in combination with tetanus toxoid (TD or Td) and whole cell pertussis (DTwP) or acellular pertussis (DTaP) formulations. Addition of aluminum salts as adjuvant increases immunogenicity of this vaccine preparations. DTwP was licensed in 1949 and DTaP — in 1981. Other combined vaccine with diphtheria toxoid may include combination of DTP with poliomyelitis virus vaccine (tetra-component formulation) or with vaccines against *Haemophilus influenzae* type B and hepatitis B (penta-component formulation).

The dosage diphtheria toxoid is measured in flocculation (Lf) units. The Lf unit is used to control quality of produced diphtheria toxoid and to confirm antigenic purity and content of toxoid prior to use in vaccine formulations [146]. It can also be used for determination antigen content in the final products. The 1st International Reference Reagent (IRR) of Diphtheria Toxoid for Flocculation Test (DIFT) was established by the WHO in 1988. This reagent is essential for the standardization of assays used to calculate Lf units of toxoids.

Vaccines for children under 7 years of age usually contain 7.5–25 Lf of toxoid per dose while vaccines for schoolchildren and adults contain 2–3 Lf per dose [44]. Diphtheria vaccine for adults is typically prepared without pertussis component (Td).

Non-toxic mutants of diphtheria toxin are considered as possible alternatives to the formaldehyde treated toxoid. These mutants called CRMs (cross-reactive materials) are naturally nontoxic and do not require chemical inactivation. Most promising antigen among

them is CRM197, which is an enzymatically inactive and nontoxic form of diphtheria toxin that contains a single amino acid substitution (G52E) in the enzymatic A subunit [147]. Currently CRM197 is used as the carrier protein in several licensed polysaccharide-protein conjugate vaccines, for example pneumococcal conjugate vaccine (Prevnar, Synflorix etc.). However, it is considered as antigen for immunization against diphtheria [148]. In addition, new vaccines based on CRM197 and directed against diphtheria are also being developed [149, 150].

Instead of diphtheria toxoid and CRM197 for immunization against diphtheria non-toxic recombinant subunits of diphtheria toxin can be considered as potential antigens to elicit immune response to distinct parts of DT molecule. Preliminary immunization with DT B-subunit was able to protect sensitive to DT animals from toxin action [95]. Recombinant B subunit had great potential to elicit protective immune response in immunized organisms, which allow considering this antigen as prospective component for future vaccine development.

The current diphtheria vaccines are delivered by parenteral route. They can induce high level of antitoxin, mainly IgG, which prevent systemic spread of the toxin. IgG antibodies also may exert a local protective effect, probably through transudation at the mucosal surfaces at site of bacterial colonization. However, IgA antibodies play more important role in the protection of mucosal surfaces of the body from mucosal-associated pathogens like *C. diphtheriae*. Mucosal vaccines can induce an immune response that more closely resembles natural immunity. In animal models of immunization, the nasal route of toxoid or CRM197 administration appears to have the advantage of inducing IgA mucosal response, making it highly attractive for the delivery of vaccines [151, 152]. However, the immunity to diphtheria in human subjects usually estimated in IU by the formation of protective serum IgG or IgM levels of immunoglobulin, but there are no accepted criteria for evaluation of toxin-neutralizing activity of secretory IgA response. This circumstance significantly slows down the progress in intranasal vaccine approval.

Vaccination schedules. The World Health Organization introduced Expanded Programme on Immunization (EPI) with the aim to make vaccination available to all children throughout the World [153, 154]. The WHO recommendation for primary immunization of infants includes administration of three doses of DTP vaccine at the age of 6, 10 and 14 weeks (WHO, 2006) [44]. Generally accepted, that after three DTP vaccinations almost all children can achieve antibody levels higher than 0.01 IU/mL [44, 155].

However, there is no universal schedule for immunization against diphtheria appropriate for each country. The choice of a right schedule depends on the epidemiological pattern of diphtheria in defined territory. In developing countries where the reservoir of *C. diphtheriae* remains large and natural immunity plays significant role in protection against the disease, the first priority of WHO is to ensure 90% coverage of infants with the primary series of three doses of DPT vaccine. In developed countries, primary immunization usually consists of 3 doses of DPT vaccine given at intervals of one month from 2nd or 3rd months of age, and boosted by a fourth dose given in the second year of life or later [44]. According to the Ukrainian schedule of immunization primary series of DPT vaccine was given at 3rd, 4th, and 5th months of age, and a booster dose was administered at 18 months of age [Order of the Ministry of Health of Ukraine № 595 from 16.09.2011 «On the procedure of vaccination in Ukraine and quality control and circulation of medical immunobiological preparations»].

Unfortunately, in developed countries antitoxin serum concentration in infants shows a dramatic decline after the primary series of vaccinations. For example, infants vaccinated against diphtheria at the ages of 3, 5 and 12 months according the Swedish vaccination schedule results incessary for long-term protection (titers 0.1 IU/ml) [156]. The decline of the antibody titers indicates a necessity of further studies to establish the duration of protection.

In countries with high rates of infant vaccination, the cases of diphtheria during childhood significantly declined. On the other hand, this leads to disappearing of toxigenic strains of *C. diphtheriae* circulating in population, which results in declining of antibody levels with age. Populations with high rates of infant vaccination can acquire susceptibility to outbreaks of diphtheria among adults, because their post-vaccination immunity decreased without permanent contact with toxigenic strains. Therefore, WHO recommend for industrialized countries to include additional boosters of DTP vaccine to the primary series of infancy immunization in order to compensate the loss of naturally acquired boosting. Various national immunization schedules propose two booster doses: one during the second year of life and a second before school entry. In addition, people living in non-endemic areas

may require additional boosters every 10 years period to retain all-time protection. Typically, a booster dose administered any time stimulates strong antitoxin production with mean levels above 1.0 IU/mL [44, 142, 157].

The Ukrainian schedule of immunization offers quite a lot of booster doses: one at 18 months of age and a second at six years of age, then at 14, 18, 23, 28 years of age and additional boosters at about 10-year intervals to maintain life-long protection [Order of the Ministry of Health of Ukraine № 595 from 16.09.2011]. Serological monitoring of antitoxin titers in population can be helpful tool for improving current immunization schedule.

Epidemiology. Economic and cultural changes including improved sanitation and hygiene may change the epidemiologic patterns of diphtheria [158]. Today diphtheria evolves from children's disease into disease affecting predominantly adults, with severe respiratory forms of infection. Outbreaks of diphtheria can reemerge until population-wide immunity will be restored by naturally acquired immunization or by broad vaccination of adults [15].

Current vaccines are very effective in preventing from severe forms of infection and infection-caused death, but they are not so effective against mild diseases or asymptomatic carrier states. Their effectiveness in protection from infection is estimated only at 70–90%. Diphtheria outbreaks still can occur among highly vaccinated populations [159, 160].

It is assumed that there is no exactly defined level of antitoxin that gives complete protection from infection, and same antitoxin titers may give diverse protection in different subjects. Thus, an antibody concentration from 0.01 to 0.1 IU/ml may be considered as basic protection, whereas a higher titer of antitoxin may be needed for complete protection. Asymptomatic diphtheria carriers show high antitoxin titers [161].

Human cases or carriers are the reservoir for this infection. In general, total immunization resulted in considerable reduction of diphtheria incidences. It also results in some changes in the immune profile of various age groups following reduction of circulation of toxigenic strains. It is believed, that circulating toxigenic strains can provide opportunities for natural boosting and maintenance of immunity to infection. Adults become more susceptible to diphtheria due to reduced opportunities to keep high immunity through subclinical infections. Protective antibody levels decreases with age, thus in some developed countries, less than 50% of adults could be immune to diphtheria. The age groups with the lowest level of diphtheria antibodies are 20–40 year old or older [15, 44]. A large pool of susceptible persons creates an epidemic potential. In some countries, old persons are still immune to diphtheria, and this is probably due to natural immunity.

Numerous studies have indicated that immunization against diphtheria toxin does not protect from the challenge of non-toxigenic *C. diphtheriae* strains. In highly immunized populations, toxigenic strains virtually disappear, although non-toxigenic strains may continue to circulate [42]. Among them the emergence of invasive non-toxigenic clones of *C. diphtheriae* [41, 162] has been described, but role of this infection as a potential source for respiratory diphtheria remains unclear.

Lessons from the diphtheria epidemic in the Former Soviet Union. Diphtheria was well controlled in the Soviet Union due to well-established childhood vaccination program initiated in the late 1950s. The huge recurrence of diphtheria at the former Soviet Union Countries was the first large-scale diphtheria epidemic in developed countries in vaccination era. Diphtheria incidence started to increase in those countries in the early 1980s, reached its first peak in 1983 to 1985 and its second peak in 1994 to 1995. Epidemic factors were a large population of susceptible adults and children due to decreased immunization coverage, terrible socioeconomic conditions and returning of the military forces from Afghanistan where diphtheria was endemic[163–165].

Due to the reappearance of the epidemic diphtheria in the Newly Independent States in 1990, the European Laboratory Working Group on Diphtheria was established in 1993 [166]. In 2006, diphtheria surveillance network has been expanded and become officially recognized by the European Commission as a dedicated surveillance network, called DIP-NET, covering 25 European countries as members. One of the main goals of this network is the evaluation and standardization of laboratory methods for diagnosis of diphtheria, especially due to the fact that diphtheria has become a rare disease in the majority of the participating countries [167].

Conclusions and Future Directions

Diphtheria was a major cause of childhood mortality in the pre-vaccination era. Routine childhood vaccination virtually eliminated diphtheria in most of industrialized countries. But outbreaks of diphtheria still occur in non-immunized and immunocompromised groups

even in developed countries. Today it is clear that high immunization coverage, prompt diagnostics and rapid identification of close contacts are principal things in controll of diphtheria outbreaks. Nevertheless, deeper understanding of the molecular mechanisms of bacterial pathogenesis is still required for efficient struggle nith the complete combating disease.

Diphtheria represents a unique model for the study of the host-microbe interaction due to phage-encoding mechanism of DT production. Most symptoms of diphtheria are resulted from the diphtheria toxin, which is a product of phage genome. Therefore, immunity against disease is antitoxin-mediated. It is believed that potent humoral immune response to DT can provide the full protection of the body against disease. Peculiarities of humoral immune response also determine current form of diphtheria infection and carrier state. However it is still unclear how the antibodies to extracellular secreted protein can help to eliminate bacterial cells. At the other hand it is apparent that antitoxic immunity could not provide protection against nontoxigenic strains, which could represent the dormant source of pathogen for the possible outbreaks of the disease.

The major characterized virulence factor of *C. diphtheriae* diphtheria toxin helps bacteria to invade the host, cause disease and evade host defense mechanisms. Years of study of the structure and function of DT have made its one of the best characterized bacterial protein toxins. But the identification of other virulence factors are still needed for complete understanding of the full picture of bacterial pathogenesis, including bacterial adhesion to the cells and spreading through the body by invasive process, biofilm formation and fibrin polymerization, intracellular viability and deal host defense mechanisms. Several experimental systems are available to clarify the mechanisms underlying *C. diphtheriae* infections: *in vivo* tests on rabbits and guinea pigs and *in vitro* tests on sensitive cell lines. Other opportunities can provide genomic information and post-genomic comparative analysis of different isolates with different pathogenic potential.

There are many important questions have been raised by recent epidemic of diphtheria in the Newly Independent States of the former Soviet Union and current outbreaks of the disease. First, this epidemic emphasized the necessities for new rapid diagnostic kits and new vaccination surveillance system for prevention of the disease and reducing the risk of the disease among children and adults in future.

Determination of anti-toxin antibodies during vaccination is essential step for the characterization of the immune status of population and monitoring long-term immunity. Such information could provide advanced recommendations for vaccination policy and can predict the spread of future diphtheria epidemics. Accurate determination of anti-diphtheria toxin antibodies is essential to establish susceptible cohorts and to obtain reliable information on the immune status of a given person in population. Therefore, it is of critical importance to develop new serological methods for this purpose that will be fast and specific.

The further understanding of the host immune response to *C. diphtheriae* will suggest novel strategies for treatment and prevention of diphtheria, along with infections caused by nontoxigenic *C. diphtheriae* strains.

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ІМУНОБІОЛОГІЯ ДИФТЕРІЇ. НОВІ ПІДХОДИ ДО ПРОФІЛАКТИКИ, ДІАГНОСТИКИ ТА ЛІКУВАННЯ ЗАХВОРЮВАННЯ

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Дифтерія є висококонтагіозним і небезпечним для життя бактеріальним токсинопосередкованим захворюванням, яке спричинюється токсигенними штамами *Corynebacterium diphtheria*, трансформованими бактеріофагом, який несе ген токсину. Збудник дифтерії та його основний фактор вірулентності — дифтерійний токсин досить добре вивчені, проте спалахи цього захворювання ще й досі виникають в усьому світі. На цей час бурхливий розвиток нових методів у галузі імунології та молекулярної біології сприяє удосконаленню профілактики, діагностики та лікування дифтерії.

В огляді висвітлено мікробіологічні, епідеміологічні, а також імунологічні аспекти дифтерійної інфекції, роль дифтерійного токсину та інших факторів вірулентності в патогенезі захворювання, роль гуморального антитоксичного імунітету в протидифтерійному захисті, а також перспективи розроблення нових діагностичних тестів, протидифтерійних вакцин, імунобіологічних препаратів та антидотів для боротьби з дифтерійною інфекцією.

Ключові слова: дифтерія, дифтерійний токсин, імунітет, діагностичні тести, вакцини, антидоти, рекомбінантні протеїни.

ИММУНОБИОЛОГИЯ ДИФТЕРИИ. НОВЫЕ ПОДХОДЫ К ПРОФИЛАКТИКЕ, ДИАГНОСТИКЕ И ЛЕЧЕНИЮ ЗАБОЛЕВАНИЯ

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Дифтерия является высококонтагиозным и опасным для жизни бактериальным токсинопосредованным заболеванием, которое вызывается токсигенными штаммами *Corynebacterium diphtheria*, трансформированными бактериофагом, несущим ген токсина. Возбудитель дифтерии и его основной фактор вирулентности — дифтерийный токсин достаточно хорошо изучены, однако вспышки этого заболевания до сих пор возникают по всему миру. В настоящее время бурное развитие новых методов в области иммунологии и молекулярной биологии способствует совершенствованию профилактики, диагностики и лечения дифтерии.

В обзоре освещены микробиологические, эпидемиологические, а также иммунологические аспекты дифтерийной инфекции, роль дифтерийного токсина и других факторов вирулентности в патогенезе заболевания, роль гуморального антитоксического иммунитета в противодифтерийной защите, а также перспективы разработки новых диагностических тестов, противодифтерийных вакцин, иммунобиологических препаратов и антидотов для борьбы с дифтерийной инфекцией.

Ключевые слова: дифтерия, дифтерийный токсин, иммунитет, диагностические тесты, вакцины, антидоты, рекомбинантные протеины.

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SOME RECENT FINDINGS IN THE BIOTECHNOLOGY OF BIOLOGICALLY IMPORTANT NUCLEOSIDES

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Some recent findings in the biotechnology of biologically important nucleosides will be discussed, *viz.*, (i) a new strategy of the cascade one-pot transformation of D-pentoses into nucleosides based on the extension and deepening of the knowledge of the mechanism of functioning of the ribokinase, phosphopentomutase, and uridine, thymidine and purine nucleoside (PNP) phosphorylases, and the role of different factors (structural, electronic, stereochemical) in the glycoside bond formation, (*ii*) the modern chemistries of the chemo-enzymatic syntheses of nucleosides, (*iii*) the transglycosylation reaction using natural and sugar modified nucleosides as donors of carbohydrate residues and heterocyclic bases as acceptors catalyzed by nucleoside phosphorylases (NP).

Key words: nucleosides, bio-mimetic synthesis, chemo-enzymatic synthesis, enzymes of nucleic acid metabolism.

Nucleosides embrace a large family of natural and chemically modified analogues of great structural diversity and a broad spectrum of biological activity. Analogues of natural nucleosides as well as nucleoside antibiotics belong to the most important classes of antiviral drugs, and they are extensively used in the treatment of a variety of cancers. Base and sugar modified nucleosides are very valuable constituents of artificial oligonucleotides of medicinal potential making these oligomers more stable in biological fluids and improving their targeting proper-ties. The chemistry of many antiviral and anticancer drugs, as well as building blocks for oligonucleotide synthesis remains a challenging problem resulting in a high price of the desired compounds preventing them from extensive therapeutic application and use in oligonucleotide business (for a recent reviews, see [1–3].

Analysis of the state of the art of chemo-enzymatic synthesis of nucleosides led us to conclusion that the chemo-enzymatic methodology demonstrates a number of advantages over the chemical methods of nucleoside synthesis, *viz.*, high total yield of desired products, simplicity of work-up of reaction mixtures and isolation of products, conform to the principles of «green chemistry» to a greater extent *vs.* the fine organic synthesis [1–3]. Up to the present, a vast majority of the modified nucleosides have been synthesized by chemical methods. Despite the impressive progress achieved in the development of chemical methods, production of many antiviral and anticancer drugs, as well as other biologically active compounds remains a challenge. This leads to high drug costs and, therefore, prevents extensive biological trials and studies, as well as a wide therapeutic application. The need for the development of new strategies became apparent in the late 1970s.

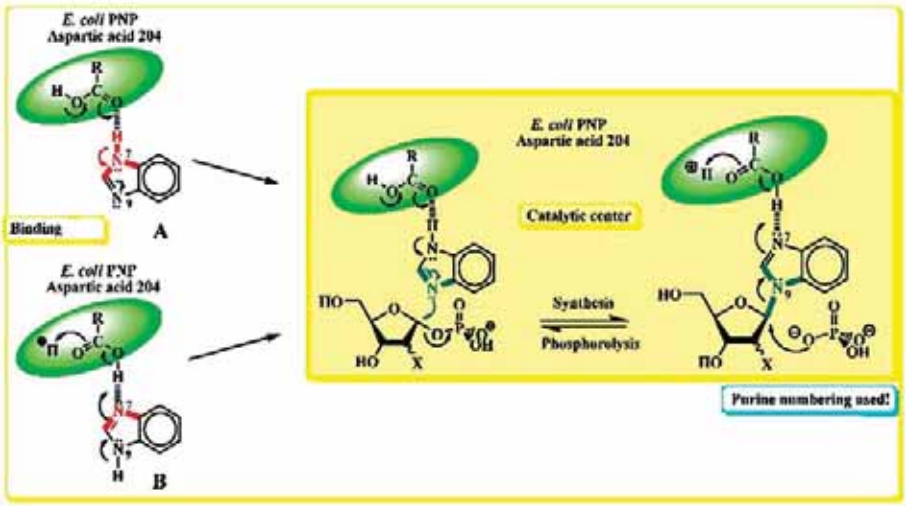
The chemo-enzymatic (biotechnological) strategies are currently displacing multi-stage chemical processes, and this allows performing the key transformations with high selectivity and *regio*- and *stereo*-specificity. Considerable progress in the production of biologically important analogs of natural nucleosides has been achieved through the rational combination of chemical and biochemical transformations. Use of recombinant nucleoside phosphorylases (NPs) and *N*-deoxyribosyl transferases (NDTs) as biocatalysts for the synthesis of natural nucleosides and their modified analogs is of considerable importance

for the creation of modern technological processes. Noteworthy that both groups of enzymes complement one another and allow finding out a straightforward route to the desired compound. The use of the chemo-enzymatic methods allows undoubtedly improve the price-quality ratio in the production of many medical drugs.

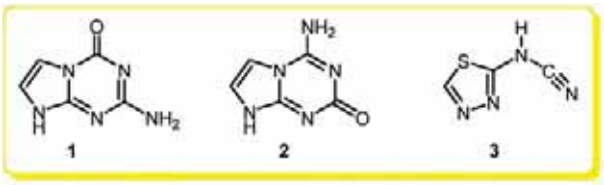
The possible areas of application of nucleoside phosphorylases for the synthesis of nucleosides, as well as the limitations of this methodology, have been investigated in detail; however, several very interesting enzymatic synthetic reactions deserve special attention, because they are crucial for understanding the mechanism of synthetic reactions catalyzed by these enzymes and may expand the scope of their practical use. It is well documented that the N^7-atom of purines plays a very important role in the phosphorolytic cleavage of the glycosyl bond of purine nucleosides and, it seems, in the reversed synthetic reaction catalyzed by *E. coli* purine nucleoside phosphorylase (PNP; product of *deoD* gene; EC 2.4.2.1) as well, even though the mechanism of this reaction has not been adequately studied. The finding that 3-deazapurines and 1-deaza-, 3-deaza- and 1,3-dideazapurines (benzimidazoles, including fluoro-, chloro- and bromo-substituted) are good substrates for PNP allows to suggest a key role for two nitrogen atoms of the imidazole ring in the synthetic reaction (for a discussion, see [1]). Namely, one of them is involved in the binding of the heterocyclic base in the enzyme's active site that may lead to an increase of the nucleophilicity of the second nitrogen atom. This facilitates, in turn, an attack by this atom on the electrophilic C1 carbon atom of α-D-pentofuranose-1-phosphate and eventually results in the formation of a glycosidic bond (Scheme 1) [2].

The mechanism of the synthetic reaction catalyzed by nucleoside phosphorylases in general and PNP in particular remains unclear. The participation of two nitrogen atoms of benzimidazole in this reaction seems to be obvious albeit there are two modes **A** and **B** of initial binding of the substrate. The Check authors studied substrate properties of a number of purine heterocyclic bases and their aza- and deaza-analogues using partially purified *E. coli* PNP and gel-entrapped cells of an auxotrophic thymine-dependent strain of *E. coli* as a biocatalyst for the transfer of the 2′-deoxy-D-ribofuranosyl moiety of 2′-deoxyuridine to bases. The reactions proceeded *regio*- and *stereo*-specifically affording purine nucleosides as well as 8-aza-2′-deoxyadenosine and 8-aza-2′-deoxyguanosine but no substrate activity of 7-deazapurines was observed. It was thus concluded that the presence of the nitrogen-7 of purines and their isosteric analogues is a prerequisite for the reaction [4]. However, there are several exceptions, *viz*., 5-aza-7-deazaguanine (**1**), 5-aza-7-deazaisoguanine (**2**) and *N*-(1,3,4-thiadiazol-2-yl)-cyanamide (**3**) are substrates for bacterial and mammalian purine nucleoside phosphorylases (PNP's) (Scheme 2) [2].

These data imply that the mechanism of binding and activation of substrate in the catalytic center of *E. coli* PNP is not uniform and prompted us to gain insight into the functioning of the enzyme and to search for new substrates and inhibitors.



Scheme 1



Scheme 2

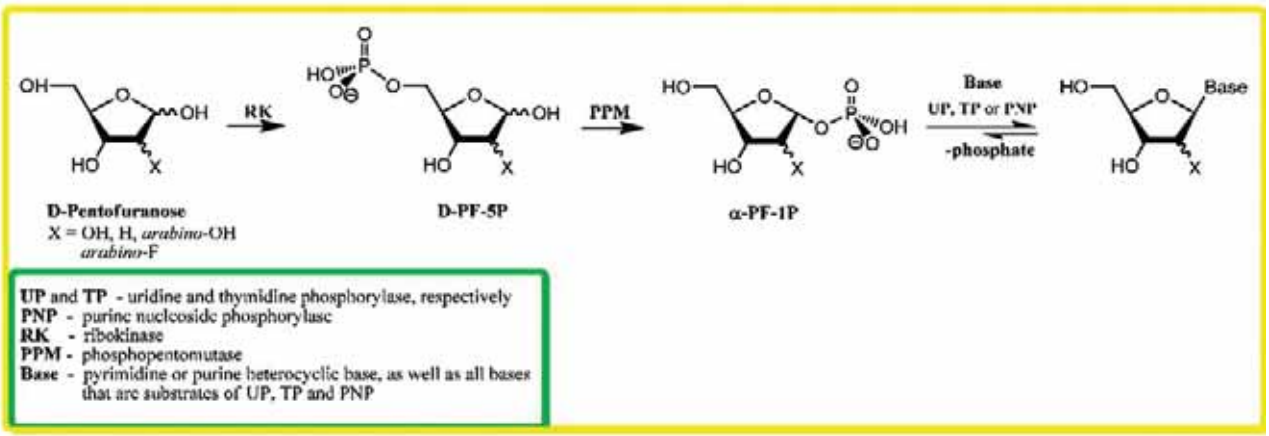
Results and Discussion

The main line of the present study based on the suggested by us new strategy of nucleoside synthesis consisting in the cascade transformation of pentoses into nucleosides through intermediate consecutive formation of a pentofuranose-5-phosphate and α-D-pentofuranose-1-phosphate (PF-α1P) catalyzed by recombinant *E. coli* ribokinase (RK), phosphopentomutase (PPM) and nucleoside (uridine, thymidine and purine nucleoside) phosphorylases (Scheme 3) [5]. Before an idea of the cascade transformation — D(L)-pentose + base → nucleoside — was proved, the chemical synthesis of PF-α1P was considered as an important supplementary approach to the preparation of sugar and base modified nucleosides for biological and medicinal application. The chemo-enzymatic nucleoside synthesis consisting of the chemical preparation of PF-α1P followed by an enzymatic condensation with heterocyclic bases seemed to be more versatile and rather attractive.

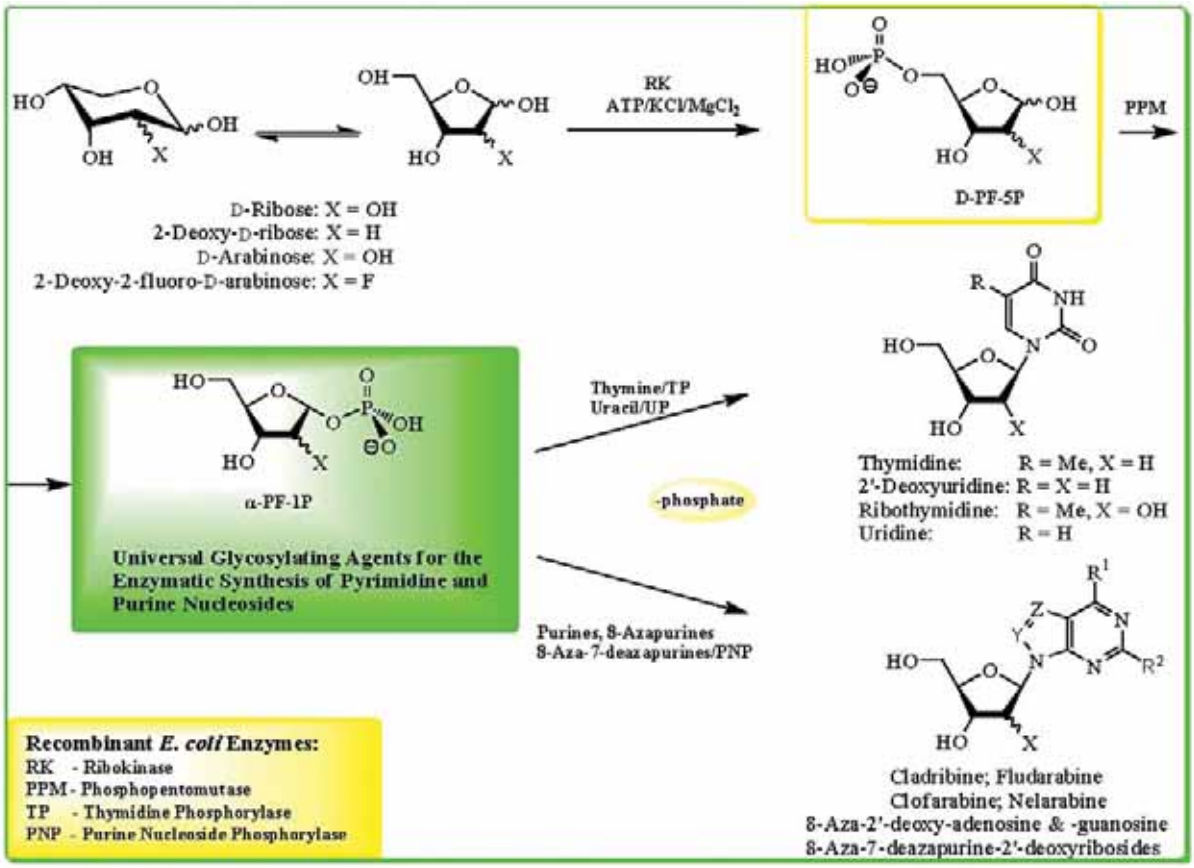
Preparation of recombinant* E. coli *ribokinase (RK), phosphopentomutase (PPM) and nucleoside phosphorylases (NP's) on the practical level is of utmost importance for the project and this task was realized and all these enzymes were obtained as they spend at work [2, 3, 5–7]. Taking into account the aims of the present study, the preparations of the aforementioned enzymes have been obtained in different forms [lyophilized powders of high enzymatic (>90% after 3-5 years storage at +4 °C) stability], solution of a protein in diverse buffers] and different levels of purity (carefully purified for biochemical studies; sufficient purity for biotechnological studies).

A possibility of cascade one-pot enzymatic transformation of D-ribose or 2-deoxy-D-ribose into nucleosides employing pure recombinant *E. coli* ribokinase (RK) [D-pentose → pentose-5-phosphate (D-PF-5P)], PPM [D-PF-5P → α-D-pentofuranose-1-phosphate (D-PF-α1P)], and nucleoside phosphorylases (NP's) (D-PF-α1P + heterobase → nucleoside) coupled with the appropriate pyrimidine or purine heterobases was demonstrated (Scheme 4) [7]. Preliminary results of a caskade transformation of D-pentoses into nucleosides pointed to a reliability to develop practical methods for the preparation of antileukemic drugs (*Cladribine*, *Fludarabine*, *Clofarabine*, *Nelarabine*) and a number of biologically important nucleosides.

We noted rather essential differences between the optimal reaction conditions for RK [5], PPM [7] and recombinant *E. coli* nucleoside phosphorylases [6]. Bearing this in mind, we have optimized the one-pot reaction conditions aiming at the finding out a compromised composition of the substrates allowing satisfactory function of the enzymes under investigation. It was found that D-ribose, 2-deoxy-D-ribose, D-arabinose and 2-deoxy-2-fluoro-D-arabinose are transformed into the respective nucleosides in good yields; D-xylose as well as some 2(3)-deoxyfluoro-D-pentofuranoses and L-pentofuranoses are not involved in the cascade transformation into nucleosides. This study is continued.



Scheme 3



Scheme 4

Our studies unambiguously showed for the first time that 1,6-diphosphates of D-hexoses are not necessary for the transformation of 5-phosphates of D-ribose, 2-deoxy-D-ribose, D-arabinose and 2-deoxy-2-fluoro-D-arabinose into the corresponding 1-phosphates. This is one of the most important findings because it essentially simplifies the application of PPM as a biocatalyst within the one-pot cascade transformation of D-pentoses into nucleosides as well as for the transformation of chemically prepared 5-phosphates into intermediary 1-phosphates and then into nucleosides (Scheme 4) [7].

The strategy of cascade one-pot synthesis of nucleosides suggested by us* [5,7] *is of interest for development of practical methods for the preparation of biologically important nucleosides. It should be emphasized that this strategy allows preparing β-D-*ribo*-, 2-*deoxy*-β-D-*ribo*-, β-D-*arabino*- and 2-*deoxy*-2-*fluoro*-β-D-*arabino*-nucleosides of natural purine and pyrimidine (except for 2-*deoxy*-2-*fluoro*-β-D-*arabino*-nucleosides) bases, as well as base modified nucleosides. Indeed, R&D of this strategy led us to the simple and efficient preparation of antileukemic drugs 2-chloro-2β-deoxyadenosine (*Cladribine*) from 2-deoxy-D-ribose and 2-chloroadenine, 9-(β-D-arabinofuranosyl)-2-fluoroadenine (*Fludarabine*) (D-arabinose + 2-fluoroadenine), 9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-2-chloroadenine (*Clofarabine*) (2-deoxy-2-fluoro-D-arabinose + 2-chloroadenine) and 2-amino-9-(β-D-arabinofuranosyl)-6-methoxypurine (*Nelarabine*) (D-arabinose + 2-amino-6-methoxypurine). Moreover, preliminary results of the synthesis of a number of biologically important nucleosides [*e. g.*, 2′-deoxyribosides of 8-azapurines and 8-aza-7-deazapurines (*vide infra*)] have validated this strategy [8].

Transfer of a pentofuranosyl moiety of commercially available nucleosides or prepared by chemical methods to purine or pyrimidine bases catalyzed by nucleoside phosphorylases (NPs) or *N*-deoxyribosyltransferases (DRTs) («transglycosylation reaction») was demonstrated to be a very efficient methodology for the synthesis of a plenty of analogues of natural nucleosides of biological and medicinal importance (for recent reviews, see, *e. g.*, [1–3]). The bacterial nucleoside phosphorylases [purine (PNP), thymidine (TP) and uridine (UP)] reversibly catalyze (*i*) the phospho-

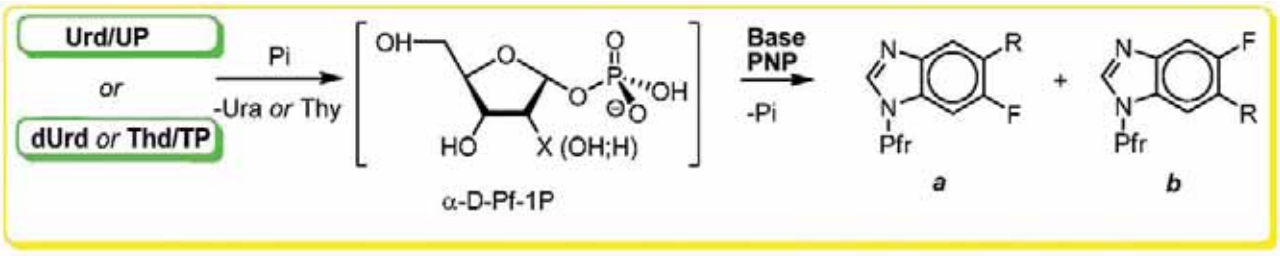
rolysis of nucleosides with an intermediary formation of α-D-pentofuranose-1-phosphate (PF-α1P), and (*ii*) the synthesis of new nucleosides in the presence of external heterocyclic bases. As distinct from nucleoside phosphorylases, DRTs catalyze the direct transfer of the 2′-deoxyribofuranosyl moiety of donors without intermediary formation of 2-deoxyribofuranose-1-phosphate. From the viewpoint of practical synthesis of sugar and base modified nucleosides, NP and DRT complement each other, but the latter have a strict specificity towards substrates thereby limiting their application.

Base halogenated nucleosides of benzimidazole attract an attention of researchers since the pioneering studies by I. Tamm and his co-workers initiated in the early 1950s of the last century (for a review, see [9]). However, the most important findings from the viewpoint of the biochemical mechanism of antiviral activity of this class modified nucleosides [10] as well as their possible practical application [11] have been published during last two decades (reviewed in [12,13]).

It was earlier shown that benzimidazole (BI) and its derivatives with substituents in the benzene ring are good substrates of *E. coli* PNP in the transglycosylation reaction [1, 14–17]. In the present project, we studied 5,6-difluorobenzimidazole and its derivatives, one fluorine atom of which is replaced with methoxy, ethoxy, isopropoxy, 4-morpholino and *N*-methylpiperazino groups, as acceptors of the D-ribofuranose and 2-deoxy-D-ribofuranose residues in the transglycosylation reaction employing uridne (Urd) and thymidine (Thd) [2′-deoxyuridine (dUrd)], respectively, as the pentofuranose donors and recombinant *E. coli* UP, TP and PNP nucleoside phosphorylases as biocatalysts [18].

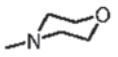
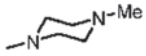
The reaction transglycosylation was employed for the synthesis of the β-D-ribo- and 2-deoxy-β-D-ribonucleosides **4**–**15**. The reaction conditions have been optimized depending on the kind of a ribofuranose donor, the donor/base ratio, and quantity of the recombinant *E. coli* enzymes and temperature of the reaction. The use of readily available natural purine ribonucleosides as donors of the ribofuranose residue in the transglycosylation reaction of BI allows employing PNP as the sole biocatalyst. On the contrary, the use of uridine as a donor of the ribofuranose residue requires two nucleoside phosphorylases for transglycosylation reactions, *viz.*, the recombinant *E. coli* uridine phosphorylase (UP) for the intermediary formation of α-D-ribofuranose-1-phosphate (D-Rib-α1P) that is accepted by *E. coli* PNP for the synthesis of the BI ribosides. The efficiency of both types of donors was tested in the transglycosylation of 5,6-difluorobenzimidazole and it was found that the use of uridine and two nucleoside phosphorylases is preferable in terms of yield of the desired nucleosides.

It was found that the 3:1 to 10:1 molar donor/base ratio and the use of 40 units of uridine phosphorylase (UP) per 1 mmol of uridine (60–160 UP units for 2′-deoxyuridine]) and 155–400 units of PNP are necessary to obtain the ribosides **4**–**15** in good yields calculated for isolated products (Table 1). Reactions were conducted at 52 °C in the *K,Na*-phosphate buffer (5–20 mM; pH 7.0) monitoring the formation of the products by HPLC, the conversion of base into nucleoside(s) was 98.5%. It is noteworthy that the synthesis of 2′-deoxyribosides was completed in 1–3 h, whereas the *trans*-ribosylation proceeded much more slowly and 22–28 h required achieving high yields of the reaction products. Similar trend was earlier observed in the case of the *ribo* and *2′-deoxyribo* nucleoside syntheses using the whole *E. coli* cells as biocatalyst (Scheme 5) [18].



Scheme 5

Table 1. **The synthesis of benzimidazole nucleosides**

Compd	Pfr [1)]	R	Yield for isolated product (%)	Ratio of isomers	
				a	b
4	Rib	-F	77	–	
5	dRib		68	–	
6	Rib	-OMe	54	95	5
7	dRib		51	41	59
8	Rib	-OEt	98	89	11
9	dRib		85	70	30
10	Rib	-O*i*Pr	75	100	–
11	dRib		65	96	4
12	Rib		75	100	–
13	dRib		80	100	–
14	Rib		79	100	–
15	dRib		68	100	–

Pfr = β-D-Pentofuranosyl (Rib — β-D-ribofuranosyl; dRib — 2′-deoxy-β-D-ribofuranosyl).

Regio-isomeric structure of all isolated nucleosides was proved by scrupulous analysis of the ^{1}H and ^{13}C NMR spectral data (incl. [^{1}H,^{1}H] & [^{1}H,^{13}C] 2D: COSY, HSQC, HMBC and NOE spectra).The predominant (OMe and OEt) or exclusive (O*i*Pr, 4-morpholino and *N*-methylpiperazino) formation of the 5-substituted 6-fluoro-1-(β-D-ribofuranosyl)-benzimidazoles was observed. The formation of the *regio*-isomeric 5-fluoro-1-(2-deoxy-β-D-ribofuranosyl)-6-methoxy(ethoxy, *i*-propoxy)benzimidazoles was observed in the trans-2-deoxyribosylation reaction of the corresponding bases. The predominant or exclusive formation of the *regio*-isomeric N^{1}-nucleosides with bulky 5-substituents of 6-fluorobenzimidazole points to a large hydrophobic pocket in the *E. coli* PNP active site that can accommodate these groups.

During recent years, the use of D-pentofuranose-1-phosphates (PF-1Ps) as substrates of an enzymatic synthesis of nucleosides attracts much attention [1–3]. It should be stressed that the enzymatic and chemical syntheses of D-ribofuranose-1-phosphate and its 2-deoxy counterpart have rather reach prehistory. However, only recently a few interesting reports from the point of view of the possible practical application have been published. There are two lines of investigation in this area, *viz*., (*i*) biochemical (microbial, enzymatic) *retro*-synthesis of 2′-deoxyribonucleosides employing the triose phosphate isomerase (TRI) and 2-deoxy-D-ribose-5-phosphate aldolase (DERA) enzymes and (*ii*) chemical synthesis of D-pentofuranose-1-phosphates (PF-1Ps) followed by the enzymatic condensation with heterocyclic bases.

Within this line of investigation, the laborious preparation of the α-D-pentofuranose-1-phosphates (PF-α1Ps) is a serious bottleneck of this approach. However, despite rather complex synthesis of PF-α1Ps this chemoenzymatic methodology for the synthesis of biologically valuable nucleosides represents an advisable alternative to an enzymatic transfer of sugar fragment of nucleoside to heterocyclic base («transglycosylation reaction») as well as to the microbial (enzymatic) *retro*-synthesis [1–3].

Scrutiny of the chemical methods for the preparation of pento(hexo)furanose-1-phosphates as well as different methods of an activation of the anomeric carbon atom shows that the most of them are laborious and low-yielding. As might be expected, the formation of anomeric mixtures was usually observed, and only the tedious crystallization-induced asymmetric transformation leads to the predominant formation of the desired dRF-α1P (reviewed in [1–3]). From the standpoint of simplicity, method suggested by MacDonald [19] for the synthesis of pyranose-1-phosphates seems to be the most efficient one and prompted us to apply it for the synthesis of α-D-arabinofuranose-1-phosphate (**19**; Ara^{F}-α1P) [20]. We focused our studies on the development of practical chemical synthesis of PF-1Ps and selected D-arabinose as a starting pentose bearing in mind that a plenty of

purine and pyrimidine β-D-arabinofuranosides manifest antiviral and anticancer activities.

It is remarkable that Ara^F-α1P synthesized by *Wright & Khorana* was found to be inactive as a substrate for the pyrimidine deoxyribose phosphorylase of *E. coli* (thymidine phosphorylase, TP) and for the purine nucleoside phosphorylase (PNP) of fish muscle [21]. These data taken together prompted us to synthesize Ara^F-α1P and to investigate its substrate properties for recombinant PNP of *E. coli*.

Treatment of the peracetyl derivative of D-arabinose **16** with anhydrous phosphoric acid under MacDonald' conditions followed by work-up gave rise to the formation of an amorphous powder or viscous oil consisting of α-D-arabinofuranose-1-phosphate (**19**; Ara^F-α1P) and β-D-arabinopyranose-1-phosphate (**20**; Ara^P-β1P) (*ca*. 50%, combined; the **19**:**20** ratio was from 1.5:8 to 1:2, according to ^{1}H NMR). The structure of both phosphates was proved by (*i*) the scrupulous analysis of the ^{1}H and ^{13}C NMR spectra as well as [^{1}H,^{1}H] and [^{1}H,^{13}C] 2D spectra of the mixtures of different anomer ratio, (*ii*) comparison with the ^{1}H and ^{13}C NMR data for the related 1-phosphates, and (*iii*) the comparative analysis of the *ab initio* calculations of 1-phosphates of selected α-D-pentofuranoses and two conformers, $_1\boldsymbol{C}^4$ and $^1\boldsymbol{C}_4$, of β-D-arabinopyranose (Table 2).

In consent with experimental results, the *ab initio* calculations point to a higher stability of both possible conformers of β-D-arabinopyranose-1-phosphate, *viz*., $_1\boldsymbol{C}^4$ and $^1\boldsymbol{C}_4$, *vs* α-D-arabinofuanose-1-phosphate [$\Delta E = E$(AP-β1P; $_4\boldsymbol{C}^1$) — E(AP-β1P; $^4\boldsymbol{C}_1$) = −11.7 kcal/mol; [$\Delta E = E$(AP-β1P;$_4\boldsymbol{C}^1$) — E(AF-α1P; O4-*exo*) = −26.9 kcal/mol] (Table 2). It was previously shown that the ratio of α- and β-anomers strongly depends on the reaction conditions implying the α/β-anomerization to the thermodynamically more stable anomer and the possibility of furanose/pyranose isomerization during the treatment of peracyl sugars with anhydrous phosphoric acid cannot be excluded. *In toto*, it appears to be rather likely the formation of β-D-arabinopyranose-1-phosphate along with the desired α-D-arabinofuranose-1-phosphate using peracetyl D-arabinofuranose as the starting compound. In this context, it is noteworthy that the *MacDonald* method was up to present study successfully employed for the synthesis pyranose-1-phosphates [19]. To escape or diminish the formation of the pyranose-1-phosphates, we focused our further studies on the preparation of new starting sugars, primary hydroxyl group of which would be protected with an acid resistant function. However, methyl 2,3,5-tri-*O*-benzoyl-α-D-arabinofuranoside (**17**) was stable under MacDonald's reaction conditions and was recovered unchanged from the reaction mixture; on the contrary, 1-O-acetyl-2,3,5-tri-*O*-benzoyl-D-arabinofuranose (**18**) showed good reactivity, but gave a mixture of the 1-phosphates **19** and **20** similar to that obtained from the peracetyl derivative of D-arabinose. In toto,

The mixture of isomeric phosphates Ara^F-α1P and Ara^P-β1P of different ratios was tested in the reaction with 2-fluoroadenine and 2-amino-6-methoxypurine catalyzed by the recombinant *E. coli* purine nucleoside phosphorylase (PNP). It was found that the pyranose phosphate Ara^P-β1P did not interfere with the reaction of the furanose phosphate Ara^F-α1P with purine bases. Moreover, the rate of formation of 9-(β-D-arabinofuranosyl)-2-fluoroadenine (*Fludarabine*) under optimum conditions [water solution (pH = 7.0); 55 °C,

Table 2. **The *ab initio* geometry optimization of α(β)-D-pentofuranose(pyranose)-1-phosphates (as mono sodium salts) (HyperChem, 8.1; *in vacuo*, basis set; medium 6-31G*)**

Compound (1-Phosphate)	Positive partial charge at the C1 carbon atom	Total (binding) energy kcal/mol	Conformation of the pentofuranose(pyranose) ring
Ribo (RF- *α* 1P)	0.425	−808 850.3	C1-exo
2-Deoxyribo (dRF- *α* 1P)	0.454	−762 140.7	C3-endo
Arabino (AF- *α* 1P) [a)]	0.464	−808 841.6	O4-exo
β-D-*Arabinopyranose*	0.410	−808 868.5	$_4$C^1(more stable)
(AP-β1P)	0.451	−808 856.8	^{4}C$_1$(less stable)

[a)]α-D-arabinofuranose-1-phosphate is thermodynamically less stable *vs* both conformers of β-D-arabinopyranose-1-phosphates, *viz*., $_4\boldsymbol{C}^1$ and $^4\boldsymbol{C}_1$; note that among the two pyranose conformers the former is more stable than the latter. Colored data are for isomeric compounds with analogous elemental composition.

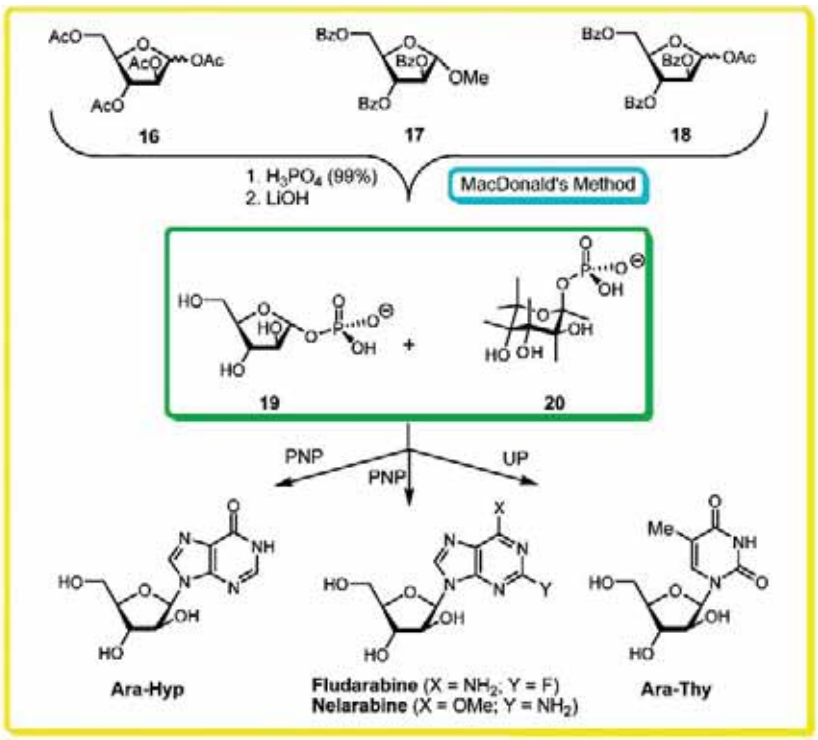
1–3 h] was found to be similar to that of the PNP catalyzed condensation of α-D-ribofuranose-1-phosphate with 2-fluoroadenine. Reaction of furanose phosphate Ara^F-α1P with 2-fluoroadenine is shifted towards the formation of *Fludarabine* and *ca*. 5% of the initial base remained after 3 h in the reaction mixture, keeping of which at 14 °C for 24 h resulted in crystallization of *Fludarabine* in 77% yield (Scheme 6) [20].

As distinct from 2-fluoroadenine, the enzymatic reaction of 2-amino-6-methoxypurine with phosphate Ara-α1P reached equilibrium at *ca*. equimolar ratio of a base and its nucleoside, 2-amino-9-(β-D-arabinofuranosyl)-6-methoxypurine (*Nelarabine*). Notably that the reaction transglycosylation of 2-amino-6-methoxypurine using 1-(β-D-arabinofuranosyl)uracil as a donor of the arabinofuranose residue and the *E. coli* uridine and purine nucleoside phosphorylases as biocatalysts gave rise to the preparation of *Nelarabine* in 53% yield [22]. Unexpectedly, 2-amino-6-methoxypurine revealed lower substrate activity compared to that of 2-fluoroadenine, whereas the respective nucleoside *Nelarabine* showed higher substrate activity for PNP *vs* *Fludarabine*. As a consequence, the reaction reached equilibrium after 36 h at *ca*. equimolar concentration of base and its nucleoside. It is noteworthy that very similar results were observed in the case of the *trans*-ribosylation [uridine as a donor of the ribofuranose residue; 23 °C; 5 mM KPB (pH 7.0)] and the *trans*-arabinosylation [1-(β-D-arabinofuranosyl)-uracil as a donor of the arabinofuranose residue; 45 °C; 5 mM KPB (pH 7.0)] of 2-amino-6-methoxypurine employing UP and PNP as biocatalysts [20].

Unexpectedly, the enzymatic reaction of hypoxanthine and a mixture of phosphates **19** and **20** (*ca*. 1:2) in the presence of PNP (172 units) in water solution (55 °C) proceeded very slowly. After 7 days, in the reaction mixture remained *ca*. 10% of the starting base (HPLC) and 9-(β-D-arabinofuranosyl)-hypoxanthine (Ara-Hyp) was isolated in 80% yield after standard work-up and chromatography.

The synthesis of 1-(β-D-arabinofuranosyl)thymine (ara-Thy) from thymine and the phosphate **19** (as a *ca*. 1:2 mixture of phosphates **19** and **20**) in water solution (55 °C) in the presence of the recombinant *E. coli* thymidine (TP) and uridine (UP) phosphorylases was studied. As expected (*cf*. [21]), TP did not catalyze the formation of the nucleoside. The formation of ara-Thy in the presence of *E. coli* UP (144 units) proceeded smoothly and *ca*. 70% of the base was transformed into ara-Thy after 7 days that was isolated in 61% yield [20].

In continuation of these studies, we investigated the chemical synthesis of 2-deoxy-2-fluoro-α-D-arabinofuranose-1-phosphate (^{2F}Ara-α1P) and its substrate properties for



Scheme 6

the recombinant *E. coli* nucleoside phosphorylases, as well as the cascade transformation of 2-deoxy-2-fluoro-D-arabinose, D-arabinose, D-xylose and D-ribose into the corresponding β-D-pentofuranosides of 2-chloroadenine. The modified MacDonald method was employed for the synthesis of ^{2F}Ara-α1P and its use as a universal glycosylating agent for the nucleoside synthesis was studied in comparison with α-Ara-1P. It was found that the phosphate α-^{2F}Ara-1P, like α-Ara-1P, is good substrate of the recombinant *E. coli* purine nucleoside phosphorylase (PNP) and can be used for the synthesis of a number of purine nucleosides (*e.g.*, *Clofarabine* and related nucleosides of 2,6-diaminopurine and hypoxanthine) as well as base modified derivatives [*e.g.*, 5-aza-7-deaza-9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)guanine]. Unexpectedly, the phosphate α-^{2F}Ara-1P, unlike α-Ara-1P, showed no substrate activity for the recombinant *E. coli* uridine phosphorylase (UP); both phosphates devoid substrate activity towards the recombinant *E. coli* thymidine phosphorylase (TP) (for a preliminary report, see [23]).

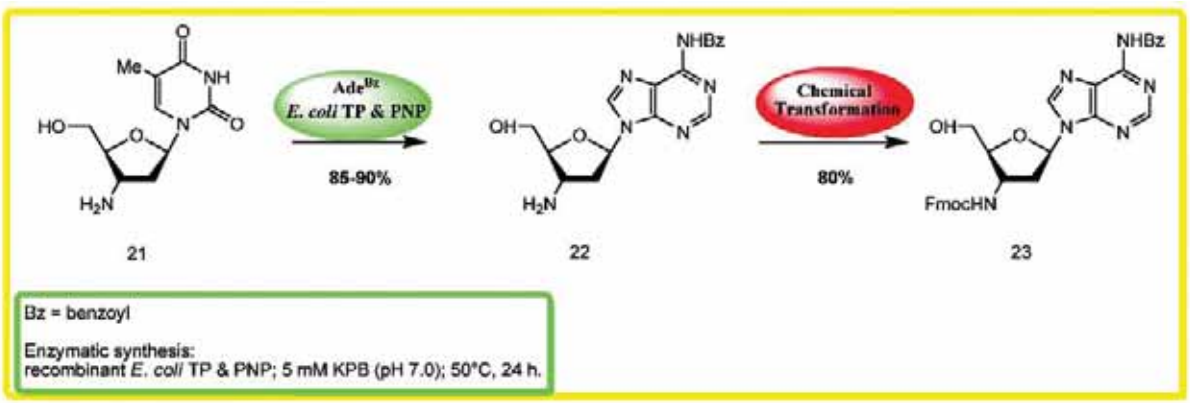
Recently we have described an enzymatic synthesis of nucleosides of N^6-benzoyladenine and N^2-acetylguanine using the respective acylated bases as acceptors of the pentofuranose residues and recombinant* E. coli *PNP as a biocatalyst [24]. Based on this finding, a new approach for the synthesis of orthogonally protected nucleosides was suggested and verified by the preparation of N^6-benzoyl-2′,3′-dideoxy-3′-Fmocaminoadenosine **(23)**. Commercially available 3′-amino-3′-deoxythymidine **(21)** and N^6-benzoyladenine were used as substrates of the enzymatic coupling catalyzed by *E. coli* PNP to give 3′-amino-2′,3′-dideoxy-N^6-benzoyladenosine **(22)** in high yield. Standard treatment of the latter with Fmoc-OSU yielded the desired nucleoside **23** with orthogonally protected amino groups of 3′-amino-2′,3′-dideoxyadenosine (Scheme 7) [24].

These studies were continued using N^2-acetyl-O^6-[2-(4-nitrophenyl)ethyl]guanine **(24)** [25] as an acceptor in (*i*) the transglycosylation reaction employing thymidine **(25)** and its 3′-aminodeoxy derivative **(26)** as donors of the pentofuranose residues and recombinant *E. coli* TP and PNP as biocatalysts (Scheme 9, path *A*), and (*ii*) the synthesis of the *ribo*- and *arabino*-nucleosides **29** and **30** in the cascade one-pot transformation of the corresponding D-ribose **(27)** and D-arabinose **(28)** into the nucleosides in the presence of the recombinant *E. coli* RK, PPM and PNP (Scheme 9, path **B**).

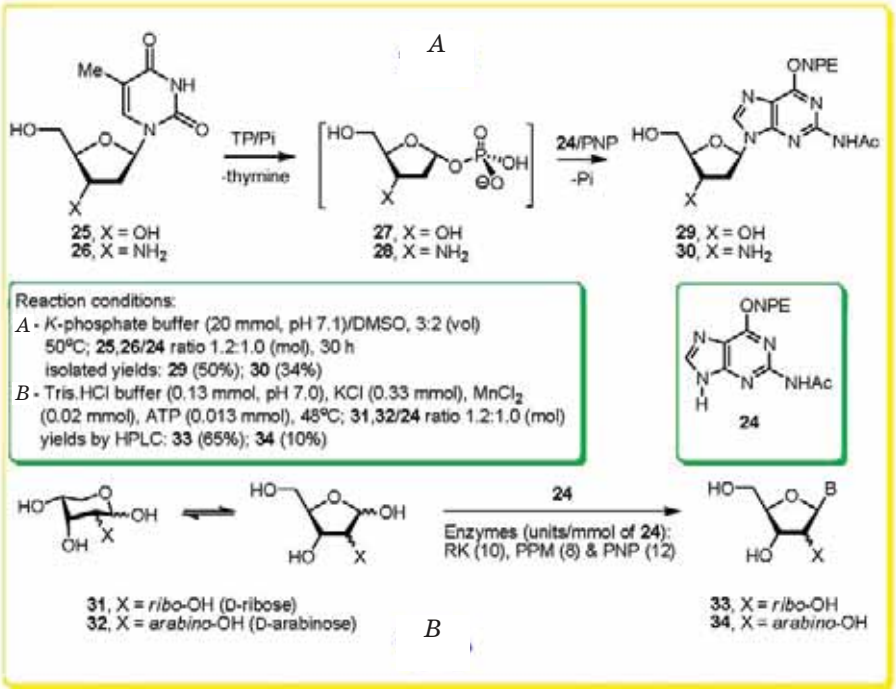
The base **24** is poorly soluble in the phosphate buffer and a 3:2 (vol) mixture of *K*-phosphate buffer (20 mM; pH 7.1) and DMSO was, therefore, used in the studied reactions. It was found that the base **24** displays satisfactory substrate activity for PNP and the nucleosides **29** and **30** form in the reaction mixture in 64 and 42% (HPLC data), respectively, after 30 h at 50 °C (Scheme 8, *A*). Both individual nucleosides have been isolated by silica gel column chromatography in 50 and 34% yields, respectively. The use of the acceptor/donor ratio of 1.0:1.5 (mol) gave rise to the formation of the nucleosides **29** and **30** in 78 and 60% yields (HPLC), respectively, after 96 h at 50 °C [26].

A good substrate activity of the base **24** for PNP prompted us to test it in the one-pot synthesis of nucleosides from D-pentoses in the cascade transformation in the presence of RK, PPM and PNP (Scheme 8, *B*). It was found that the riboside **33** forms in the reaction mixture in 65% yield after incubation for 48 h at 48 °C. Under similar reaction conditions, the formation of the arabinoside **34** proceeds more slowly affording 10% of the product after 48 h.

The finding that base **24** with voluminous NPE group shows good substrate activity points to a large hydrophobic pocket in PNP



Scheme 7



Scheme 8

active site that can accommodate this group. The dimensions of this pocket are illustrated by the overlay of imidazole fragments of the geometry optimized structures of N^2-acetyl-O^6-[2-(4-nitrophenyl)ethyl]guanine and 5-morpholino-6-fluorobenzimidazole (*vide supra*) (Fig. 1).

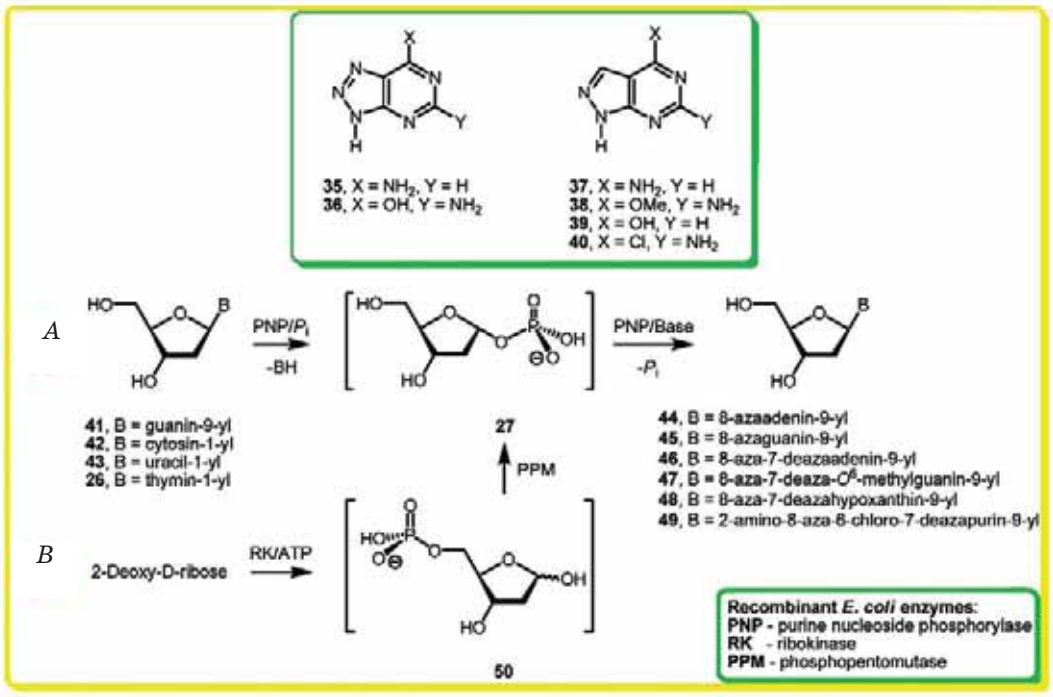
Remarkably, the pocket in the E. coli PNP active site exceeds the dimension of purine base and allows suggesting a minimal contribution of the C6 amino/carbonyl groups and N1 atom to the substrate binding.

Nucleosides of 8-azapurines and 8-aza-7-deazapurines (purine numbering throughout) are applicable as drugs and tools in chemistry, chemical biology and molecular diagnostics (see, e.g., [27–31]). Chemical synthesis of these nucleoside shape mimics suffers from the formation of mixtures of regio-isomers and α/β-anomers in the case of the synthesis 2-deoxy-D-pentofuranosides and arabinosides. This makes it necessary to conduct time-consuming separation. As a result, the desired nucleosides are obtained in moderate or low yields (e.g., [29,31]).

We applied the enzyme catalyzed glycosylation to diversity of heterocyclic bases belonging to the classes of 8-azapurines (35 & 36) and 8-aza-7-deazapurines (37–40) using various donors of the 2′-deoxy-D-ribofuranosyl residue and recombinant E. coli PNP as a biocatalyst (transglycosylation reaction; Scheme 9; path *A*) and the enzymatic cascade transformation of 2-deoxy-D-ribose into the nucleosides (Scheme 10; path B) using recombinant E. coli RK, PPM and PNP (one-pot synthesis). The enzymatic synthesis of N9-2′-deoxy-β-D-ribonucleosides of a number of 8-azapurines (44, 45) and 8-aza-7-deazapurines (46–49) was studied (Scheme 9) [8].

At first, the substrate activity of bases 35-39 was tested in the transglycosylation reaction performed under standard conditions using 2′-deoxyguanosine (41) as a glycosyl donor (Scheme 10, *A*). All these bases are satisfactory substrates and the respective nucleosides were obtained in good yields. The structure of the nucleosides 44–48 was confirmed by 1H- and 13C-NMR spectroscopy data and UV spectra as well by comparison with already published spectral data. We have not observed the formation of the regio-isomeric nucleosides.

In the next series of experiments, we studied the one-pot synthesis of nucleosides 44–48 with 2-deoxy-D-ribose and heterocyclic bases 35-39 in the presence of recombinant RK, PPM and PNP (Scheme 10; path *B*). Under reaction conditions employed, the formation of 8-aza-2′-deoxyadenosine (44) and 8-aza-2′-deoxyguanosine (45) proceeded slowly affording the nucleosides in moderate yields. On the contrary, 8-aza-7-deazapurines 37–39 showed satisfactory substrate activity and the respective nucleosides 46–48 were formed in yields more than 50% after 20 h [8].



Scheme 9

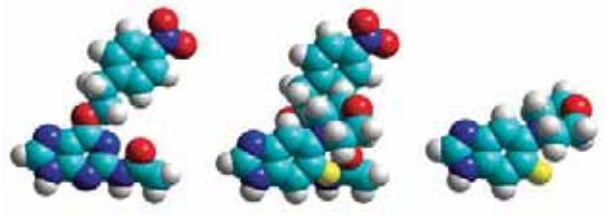


Fig. 1.

The regio-specific 2′-deoxy-D-ribosylation of 8-azaadenine (35) and 8-azaguanine (36) catalyzed by recombinant E. coli PNP is in line with published data [32]. On the contrary, glycosylation of anions of 8-azapurines with 2-deoxy-3,5-di-O-(4-toluoyl)-α-D-erythro-pentofuranosyl chloride gave a very complex mixture of regio-isomers and their α,β-anomers [28, 29].

From a chemical point of view, the formation of the glycosyl bond results from a nucleophilic attack of a nitrogen atom of base at the electrophilic C1-carbon atom of 2-deoxy-D-ribofuranose-1-phosphate (27). The regioselectivity of the enzymatic glycosylation is governed by the binding mode of base in the catalytic center of the enzyme enabling a nucleophilic attack of a nitrogen atom of the sp2 hybridized imino $-C=N^{9}-$ tautomer at the electrophilic center of co-substrate (Scheme 1). A similar type of binding of the substrate can be tolerated with a high probability for natural purine bases as well as for 8-azapurines 35 and 36 leading to the exclusive formation of N9-glycosides. However, the formation of nucleosides 46–48 was rather unexpected in the light of the earlier data pointing to the crucial importance of the nitrogen-7 of purines and their isosteric analogues in the synthetic reaction catalyzed by E. coli PNP [2, 3, 32].

Detailed analysis of the mechanism of the phosphorolysis of purine nucleosides by *E. coli* PNP showed that the base binding site is formed mainly by Asp204 and to some extent by Phe159 [33]. Taking into account that this is an equilibrium reaction, one can expect that the same amino acid residues make the main contribution in binding of substrate in the synthetic reaction. The Asp204 interacts with the nitrogen-7 and likely the C6 substituents of natural purine bases giving rise, in all likelihood, to the proper base orientation and to enhancement of the nucleophilic properties of the nitrogen-9, i.e., activation of the substrate. It is, therefore, surprising that replacement of the nitrogen-7 with CH group did not abolish the substrate activity of bases 37–39 pointing to the rather efficient contribution of the Asp204-C6 substituent interaction in the correct binding and activation [1]. To prove the role of such interaction, we investigated the substrate properties of base 40, which has no groups (NH_2, carbonyl, OH or

OR) that could imitate an interaction of natural purine substrates with Asp204. It was surprising to found that base 40 still retains moderate substrate activity despite the absence of any interactions with Asp204 and the extremely low solubility.

Analysis of the crystal structure of the ternary complex of hexameric *E. coli* PNP with Formycins A and B showed that the Ser90 is involved in binding of the bases [33a,b]. Moreover, crystallographic data for the adenine binding to the active site of *E. coli* PNP clearly showed a close proximity of Ser90·O^{γ} to the carbon-8 of the base [33c,d]. These data together with the aforementioned considerations suggest the possible explanation for the good substrate properties of bases 46–48 and moderate activity of base 49, viz., Ser90·O^{γ} is hydrogen bonded to nitrogen-8 (purine numbering) of the bases giving rise to the acceptable base orientation in the catalytic site of E. coli PNP followed by activation of the nitrogen-9 in productive complex 27 (Fig. 2). Contribution of Phe159 of E. coli PNP in both processes, binding and activation, appears to be analogous to that of the natural bases. Thus, in the case of 8-aza-7-deaza purine analogs Ser90 residue of the catalytic site of E. coli PNP takes effect of Asp204 in the case of natural purine substrates.

The spatial tautomeric structures of base 40 in complex with Ser90·O^{γ} have been analyzed by the restricted Hartree-Fock (RHF) ab initio method using basis set of 6-31** FIREFLY QC package, which is partially based on the GAMESS (US) source code. The files of MOPAC format containing Z-matrix of internal coordinates obtained by the PM3 geometry optimization was used as starting approximation for the ab initio calculations. The following main dimensions were obtained for the respective structure E. coli PNP/base 40 — Ser90-O^{γ}–H···N^{8} 0.97 & 1.86 E; Ser90-O^{γ}···H–N^{9} 2.39 & 0.99 E; =N^{8}–N^{9}(H)- 1.39 E; 27 — Ser90-O^{γ}···H–N^{8} 2.37 & 0.99 E; Ser90-O^{γ}–H···N^{9} 0.96 & 1.83 E; –N^{8}(H)–N^{9} = 1.37 E; these data are in fair agreement with strong hydrogen bonding of the base enabling the correct binding followed by activation [8].

To prove this suggestion, the Ser90Ala mutant of *E. coli* PNP was prepared and its catalytic activity in the synthesis of purine and 8-aza-7-deazapurine nucleosides studied.

The transribosylation of hypoxanthine and 8-aza-7-deazahypoxanthine (allopurinol) using uridine and the recombinant *E. coli* uridine phosphorylase for the generation of

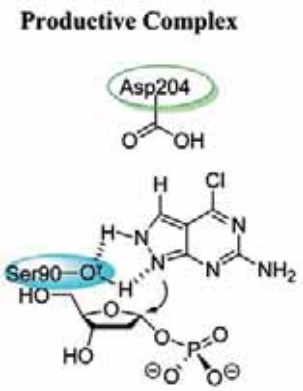


Fig. 2. **Schematic presentation of productive *E. coli* PNP/base 40 complex**

intermediary α-D-ribofuranose-1-phosphate revealed the differences in rates of the formation of inosine and allopurinol riboside (AR) along with high yields of both nucleosides. In the presence of the native E. coli PNP, the formation of (i) inosine reached 90% after ca. 30 min and after next 30 min established an equilibrium at ca. 2:8 base — nucleoside ratio in the reaction mixture, and (ii) 8-aza-7-deaza-9-(β-D-ribofuranosyl)purine (AR) proceeded somewhat slowly achieving a 95% conversion of allopurinol to its N^{9}-riboside 3a after 24 h, witnessing to non-critical role of the N^{7}-nitrogen atom in the binding and activation of heterocyclic substrate in the synthesis of nucleosides catalyzed by the native *E. coli* PNP. Replacement of the native PNP with the mutated enzyme in the synthetic reactions resulted in (i) the slowing down of the rate of the inosine formation (35% yield of inosine after 1 h; an equilibrated ca. 2:8 mixture of base — nucleoside after 24 h), and (ii) dramatic reduction of the rate of the AR formation attaining ca. 20% yield after 48 h. These data allow implying the moderate contribution of the Ser90 residue of the catalytic center of the native E. coli PNP in the binding of α-D-Rib-1P. Dramatic reduction of the rate of the allopurinol riboside formation points to an unique importance of Ser90 residue of the native *E. coli* PNP in the binding and activation of allopurinol as well as other 8-aza-7-deazapurines [especially 2-amino-8-aza-6-chloro-7-deazapurine (40)] in the enzymatic synthesis of their nucleosides (for a preliminary report, see [34].

Thus, the pure recombinant E. coli ribokinase (RK), phosphopentomutase (PPM) and nucleoside phosphorylases [uridine (UP), thymidine (TP) and purine nucleoside (PNP)] were prepared on a multigram level [5–7].

A new strategy for the synthesis of nucleosides consisting in one-pot enzymatictransformation of D-ribose, 2-deoxy-D-ribose, D-arabinose and 2-deoxy-2-fluoro-D-arabinose in the presence of heterocyclic bases into the pyrimidine and purine nucleosides was suggested and validated. It consists in consecutive transformation of D-pentoses into nucleosides under the action of recombinant E. coli ribokinase (RK) [D-pentose pentose-5-phosphate (D-PF-5P)], PPM [D-PF-5P α-D-pentofuranose-1-phosphate (D-PF-α1P)], and nucleoside phosphorylases (NPs) (D-PF-α1P + heterobase nucleoside). It was unambiguously shown for the first time that 1,6-diphosphates of D-hexoses are not necessary for the transformation of 5-phosphates of D-pentoses into the corresponding 1-phosphates. Practical methods for the synthesis of antileukemic drugs (Cladribine, Fludarabine, Nelarabine, Clofarabine) and a number of biologically important nucleosides were developed [7].

The enzymatic trans-ribosylation and trans-2-deoxyribosylation was used for the synthesis of the corresponding nucleosides of 5,6-difluorobenzimidazole and its derivatives, one fluorine atom of which is replaced with methoxy, ethoxy, isopropoxy, 4-morpholino and N-methylpiperazino groups aiming at search of new biologically active nucleosides. A large pocket close to the area corresponding to the purine N^1 atom was disclosed [18].

The MacDonald' method was studied for the synthesis of α-D-arabinofuranose-1-phosphate (Ara^F-α1P) using diverse starting peracyl derivatives of D-arabinose. Mixtures of different ratios of Ara^F-α1P and β-D-arabinopyranose-1-phosphate (Ara^P-β1P) were obtained and they will be used in the enzymatic condensations with purine and pyrimidine bases giving rise to the nucleosides, incl. Fludarabine, Nelarabine and 1-(β-D-arabinofuranosyl)-thymine. Similar approach was studied for the synthesis of antileukemic drug 9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-2-chloroadenine (Clofarabine) [20,23].

An enzymatic synthesis of 2′-deoxyribonucleosides of 8-azapurines and 8-aza-7-deazapurines has been studied using the transglycosylation reaction and cascade one pot synthesis from 2-deoxy-D-ribose and nucleobases. Good substrate activity of 8-aza-7-deazapurinestowards recombinant E. coli purine nucleoside phosphorylase (PNP) was disclosed and mechanism of binding and activation of these analogs in the catalytic site of E. coli PNP was studied. The participation of $Ser90{\cdot}O^{\gamma}$ of *E. coli* PNP in the binding of 8-aza-7-deazapurines in the catalytic center of PNP followed by the formation of productive complex and glycosidic bond was suggested [8] and proved by the preparation of the Ser90Ala mutant of *E. coli* PNP and investigation of its substrate properties [34].

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ДЕЯКІ НОВІ ДАНІ БІОТЕХНОЛОГІЇ БІОЛОГІЧНО АКТИВНИХ НУКЛЕОЗИДІВ

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Обговорено нещодавні відкриття в галузі біотехнології біологічно активних нуклеозидів, до яких належить низка лікарських препаратів проти лейкемії (кладрибін, флударабін, неларабін, клофарабін). Докладно розглянуто: нову стратегію каскадної «one-pot» трансформації D-пентоз у нуклеозиди, що ґрунтується на розширенні та поглибленні знань про механізм функціонування рибокінази, фосфопентомутази, уридин-, тимідин- і пуриннуклеозидфосфорилаз, а також роль різних чинників (структурних, електронних, стереохімічних) у формуванні глікозидного зв'язку; сучасні методи хемоензиматичного синтезу нуклеозидів; реакцію трансглікозилювання, що каталізується нуклеозидфосфорилазами, за якої донорами карбогідратних залишків виступають природні та модифіковані за цукрами нуклеозиди, а акцепторами — гетероциклічні основи.

Ключові слова: нуклеозиди, біоміметичний синтез, хемоензиматичний синтез, ензими метаболізму нуклеїнових кислот.

НЕКОТОРЫЕ НОВЫЕ ДАННЫЕ БИОТЕХНОЛОГИИ БИОЛОГИЧЕСКИ АКТИВНЫХ НУКЛЕОЗИДОВ

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Обсуждаются недавние открытия в области биотехнологии биологически активных нуклеозидов, к которым относится ряд лекарственных препаратов против лейкемии (кладрибин, флударабин, неларабин, клофарабин). Подробно рассмотрены: новая стратегия каскадной «one-pot» трансформации D-пентоз в нуклеозиды, основанная на расширении и углублении знаний о механизме функционирования рибокиназы, фосфопентомутазы, уридин-, тимидин- и пуриннуклеозидфосфорилаз, а также роль различных факторов (структурных, электронных, стереохимических) в формировании гликозидной связи; современные методы хемоэнзиматического синтеза нуклеозидов; катализируемая нуклеозидфосфорилазами реакция трансгликозилирования, при которой в качестве доноров карбогидратных остатков выступают природные и модифицированные по сахарам нуклеозиды, а акцепторов — гетероциклические основания.

Ключевые слова: нуклеозиды, биомиметический синтез, хемоэнзиматический синтез, энзимы метаболизма нуклеиновых кислот.

UDK 57.083.2+616.523

ПОИСК ИНГИБИТОРОВ РЕПЛИКАЦИИ ВИРУСА ГЕРПЕСА: 30 ЛЕТ ПОСЛЕ АЦИКЛОВИРА

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В обзоре осуществлен анализ изучения и применения различных химических соединений в качестве ингибиторов репликации вируса герпеса. Однако он не претендует на полное изложение всех материалов по поиску активных антигерпетических препаратов. После открытия первого антигерпетического препарата — ацикловира — прошло 30 лет. За это время появилось много активных химических соединений, которые послужили основой для создания антивирусных препаратов, достигнуто существенное понимание стратегии поиска препаратов, результатом которого стало, в частности, создание депо-форм антигерпетических препаратов. На основании огромного объема опубликованного экспериментального материала авторы делают вывод о том, что изучение вируса герпеса и поиск ингибиторов его репликации остается доныне важной проблемой и требует совместных усилий химиков, биологов и фармацевтов.

Ключевые слова: вирус герпеса, противовирусные препараты, ацикловир.

Герпетическая инфекция человека охватывает не менее 80% населения земного шара, а ее наиболее частая форма — генитальный герпес, имеющий выраженный рецидивирующий характер течения, получил повсеместное распространение и является одной из важнейших проблем как в России, так и за рубежом. Кроме того, герпетическая инфекция практически всегда присутствует у людей, инфицированных вирусом иммунодефицита человека (ВИЧ), что осложняет течение вызываемого им СПИДа и приводит к быстрому летальному исходу [1]. Среди ВИЧ-инфицированных пациентов, коинфицированных вирусом герпеса, около 6–10% имеют штаммы вируса, резистентные к имеющимся антигерпетическим препаратам. На сегодняшний день идентифицировано восемь типов герпесвирусов, заражающих человека: вирусы простого герпеса (ВПГ) 1-го и 2-го типа, вирус ветряной оспы (тип 3), вирус Эпштейна-Барр (тип 4), цитомегаловирус человека (тип 5), розеоловирусы (ВГЧ-6А, 6В и 7) и герпесвирус человека 8-го типа (ВГЧ-8), ассоциированный с саркомой Капоши. Вирус Эпштейна-Барр и ВГЧ-8 являются онкогенами [2, 3]. Для представителей семейства Herpesviridae характерны следующие общие свойства:

1. Геномы вирусов кодируют значительное количество энзимов метаболизма нуклеиновых кислот (тимидинкиназу, рибонуклеотидредуктазу, ДНК-полимеразу, хеликазу, праймазу) и протеинкиназу.

2. Синтез ДНК и сборка вирусного капсида происходят в ядре, созревание вириона — в цитоплазме.

3. Литический характер вирусов, т.е. образование нового инфекционного поколения, сопряжено с разрушением клетки.

4. Способность поддерживать в клетках хозяина латентную инфекцию. Латентный геном сохраняет способность реплицироваться и вызывать болезнь при рецидивной инфекции.

Большинство современных препаратов для лечения герпетических инфекций основаны на использовании в качестве лекарственных средств модифицированных нуклеозидов или их депо-форм [4]. Действие этих препаратов направлено главным образом на подавление активности вирусной ДНК-полимеразы. Следует отметить, что препараты не избавляют пациентов от рецидивирующего характера течения болезни,

а результатом их длительного приема может стать возникновение резистентных штаммов вируса. Эти обстоятельства делают актуальным поиск новых антигерпетических препаратов и их мишеней. В обзоре приведены данные по использованию клинически одобренных антигерпетических препаратов, а также по поиску новых эффективных и малотоксичных веществ, подавляющих репликацию ВПГ-1 человека, который ведется, в том числе, и в Институте молекулярной биологии им. В. А. Энгельгардта РАН.

Клинически одобренные антигерпетические препараты

Аналоги нуклеозидов и их депо-формы играют основную роль при создании антигерпетических препаратов [5]. Первый антигерпетический препарат нуклеозидной природы — 5-йодо-2′-дезоксиуридин, использовавшийся в клинической практике для лечения герпесного кератита, появился в конце 50-х годов XX века. В следующие два десятилетия для терапии были одобрены трифтортимидин, аденинарабонозид и бромвиниддезоксиуридин, также являющиеся аналогами нуклеозидов. Эти препараты, однако, имели низкую селективность и проявляли достаточно высокую токсичность, вследствие чего их использовали только для наружного применения. Второе поколение антигерпетических препаратов было создано на основе ациклических нуклеозидов (ацикловир, валацикловир, ганцикловир, пенцикловир, фамцикловир), подавляющих инфекции, вызываемые HSV-1, HSV-2, VZV и цитомегаловирусом [6–10].

Ацикловир и валацикловир

Ацикловир (ACV, первоначальное название — ациклогуанозин) был впервые синтезирован компанией Burroughs Wellcome в рамках программы по поиску гуанозиновых нуклеозидов, устойчивых к действию фосфорилаз. С появлением ацикловира (рис. 1, *a*) началась новая эра в развитии антивирусной химиотерапии.

Ацикловир оказался эффективным и малотоксичным препаратом. Механизм действия ацикловира основан на его фосфорилировании вирусной тимидинкиназой с образованием соответствующего монофосфата (ACVMP). Последующие две стадии фосфорилирования катализируют киназы клетки-хозяина с образованием трифосфата (ACVTP), который является субстратом вирусной ДНК-полимеразы, включается в цепь вирусной ДНК и блокирует ее синтез [11]. До настоящего времени ацикловир является золотым стандартом при поиске антигерпесных препаратов [12], а автор его разработки, Гертруда Элайн, получила Нобелевскую премию по физиологии и медицине в 1988 году.

Недостатком ацикловира были низкая биодоступность, плохая растворимость и короткое время жизни препарата в крови. В связи с этим для поддержания необходимой концентрации ацикловира в крови пациентов необходимы были достаточно большие дозы и частое введение препарата, что, в свою очередь, вызывало повышение токсичности. Оральная биодоступность ацикловира составляет, согласно разным источникам, 10–20%, а растворимость — около 0,2%. Для улучшения растворимости и повышения биодоступности были синтезированы депо-формы ацикловира, а именно 2′-O-глицил- и 2′-O-аланилацикловир [4]. Однако при клинических исследованиях препараты проявили достаточно высокую токсичность.

Эффективным и безопасным препаратом оказался валиновый эфир ацикловира, валацикловир (рис. 1, *b*). Повышенная оральная биодоступность валацикловира, вероятно, является результатом быстрой

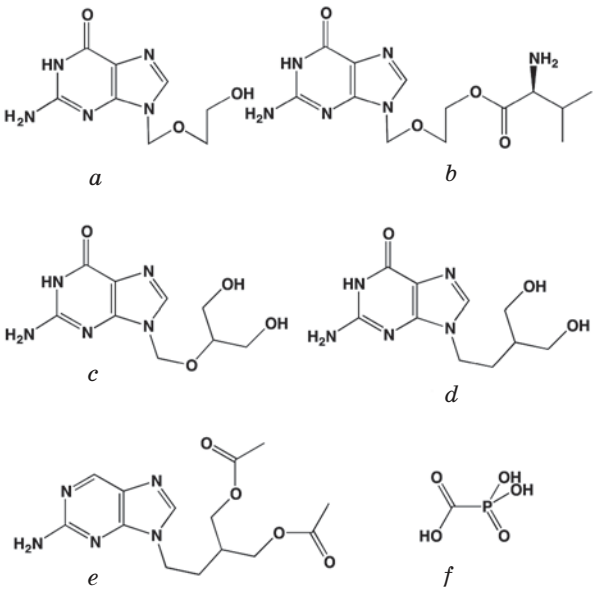


Рис. 1. **Структурные формулы противогерпетических препаратов, применяемых в клинике: ацикловир (*a*), валацикловир (*b*), ганцикловир (*c*), пенцикловир (*d*), фамцикловир (*e*), фоскарнет (*f*).**

кишечной абсорбции, регулируемой транспортером пептидов 1 (hPEPT1), и последующего эффективного превращения препарата в ацикловир в результате гидролиза в тонком кишечнике [4].

Одна из последних описанных в литературе модификаций ацикловира, которая существенно повышала его биодоступность, — биотинилирование. Соединения, содержащие биотиновую группировку, остаток ацикловира и гидрофобный линкер, транспортировались в клетку натрийзависимым транспортером мультивитаминов (SMVT). Скорость накопления таких веществ в клетке была приблизительно в 13 раз выше, чем ацикловира, при такой же цитотоксичности [13].

Ганцикловир

Ганцикловир (GCV) — 9-(1,3-дигидрокси-2-пропоксиметил)гуанин — ациклический аналог гуанозина (рис. 1, *c*), структура которого близка к структуре ацикловира [14]. Препарат значительно эффективнее ацикловира относительно цитомегаловируса (CMV) и используется для лечения вызванных им заболеваний у пациентов с ослабленным иммунитетом, в частности при цитомегаловирусном ретините (воспаление сетчатки глаза) у больных СПИДом. Как и в случае ацикловира, оральная биодоступность ганцикловира низка, и, чтобы преодолеть эту проблему, впоследствии был синтезирован валганцикловир, валиновый эфир ганцикловира [15].

Пенцикловир и фамцикловир

Пенцикловир (PCV, рис. 1, *d*) представляет собой ациклический аналог гуанозина со структурой, близкой структурам ацикловира и ганцикловира, но без кислорода в ациклической «сахарной» части и с ОН-группой в позиции, эквивалентной 3′-ОН-группе природного дезоксинуклеозида. Впервые PCV был синтезирован в лабораториях Beecham Pharmaceuticals. В экспериментах на клеточных линиях препарат был менее активен против ВПГ-1 (IC_{50} = 0,4 ± 0,1 мкг/мл), чем ацикловир (IC_{50} = 0,2 ± 0,2 мкг/мл), но эффективно подавлял репликацию ВПГ-3 (IC_{50} = 3,1 ± 0,8 мкг/мл). Интересно, что при высокой множественности инфицирования клеток вирусом PCV был активнее, чем ACV, и, в отличие от ацикловира, подавлял развитие штамма ВПГ-1, несущего ген мутантной ДНК-полимеразы (IC_{50} = 1,5 мкг/мл) [16].

Аналогично ацикловиру пенцикловир превращается в монофосфат вирусной тимидинкиназой. Первоначальное превращение PCV в пенцикловирмонофосфат более эффективно, чем фосфорилирование ACV, однако трифосфат пенцикловира, образующийся в инфицированных клетках, является менее эффективным ингибитором синтеза вирусной ДНК, чем ACVTP, а его оральная биодоступность ниже, чем у ацикловира. Для улучшения свойств препарата была синтезирована его депо-форма — фамцикловир (рис. 1, *e*), являющийся диацетильным производным пенцикловира [4]. Фамцикловир превращается в пенцикловир *in vivo* под действием эстеразы, катализирующей удаление ацетильных групп, и альдегидоксидазы, окисляющей пуриновое основание. Следует отметить, что в культурах тканей фамцикловир не обладает противовирусной активностью, так как в них не происходит окисления пенцикловира, но многочисленные исследования *in vivo* показали, что при пероральном применении он эффективно подавляет ВПГ в различных животных моделях. В экспериментальной инфекционной модели на мышах с подавленным иммунитетом фамцикловир оказался более эффективным, чем ацикловир в элиминации вируса из пораженных органов (ухо, мозг). Еще одним из преимуществ фамцикловира является его способность предотвращать латентную инфекцию ВПГ-1. Было показало, что значительно меньше латентного вируса обнаруживается в ганглиях мышей, которых лечили фамцикловиром, по сравнению с валацикловиром. Причины предотвращения рецидивной инфекции фамцикловиром в условиях, когда валацикловир не приводит к таким результатам, остаются пока без объяснения.

Фоскарнет

Фоскарнет (PFA) (рис. 1, *f*) представляет собой аналог пирофосфата и является ненуклеозидным ингибитором ДНК-полимеразы ВПГ. Фоскарнет неконкурентно (по отношению к нуклеотидам) связывается с активным центром энзима и имитирует уходящую пирофосфатную группу, препятствуя связыванию входящего нуклеозидтрифосфата [17]. PFA применяется в клинике только когда лечение ацикловиром и другими нуклеозидными препаратами невозможно в связи с возникшей резистентностью к нуклеозидным препаратам. PFA подавляет синтез вирусной ДНК при концентрациях, сопоставимых с таковыми для ацикловира, однако он обладает значительно более высоким уровнем цитотоксичности, вызывает побочные эффекты и должен применяться внутривенно, а не перорально [18].

Поиск новых антигерпетических препаратов

В литературе описан ряд новых интересных производных нуклеозидов, обладающих противогерпетической активностью, например, ациклический H2G (рис. 2, *a*) и карбоциклические циклобутановый (лобукавир) (рис. 2, *b*) и циклопропановый (А-5021) аналоги гуанина (рис. 2, *c*). Однако после клинических испытаний соединения не были одобрены как лекарственные препараты из-за повышенной токсичности [19].

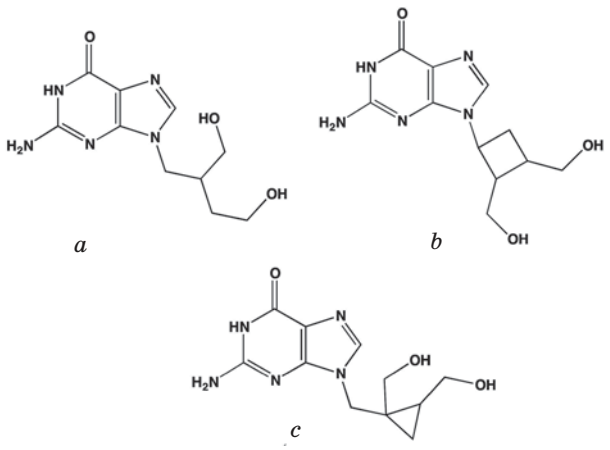


Рис. 2. **Структурные формулы : H2G (*a*), лобукавир (*b*), А-5021 (*c*)**

Фосфонатные производные нуклеозидов

В настоящее время в клинике используют три фосфонатных производных ациклических нуклеозидов, которые активируются в клетке, минуя стадию первичного фосфорилирования: аналог цитидина сидофовир, используемый для лечения цитомегаловирусной инфекции, и аналоги аденина адефовир и тенофовир, применяемые в терапии гепатита В и ВИЧ, соответственно. Противогерпетическое действие фосфонатных производных ацикловира ранее описано не было, в связи с чем исследования потенциальной активности фосфонатных производных ACV и других нуклеозидов представляли определенный интерес.

В нашей лаборатории были синтезированы фосфонатные (Z)- и (E)-изомеры 9-[3-(фосфонометоксипроп)-1-ен-1-ил] аденина (14) и фосфонатный аналог ацикловира (фосфит ацикловира, HpACV) (рис. 3) [20].

Ациклические фосфонатные аналоги аденина (рис. 3, *b* и *c*) ингибировали репликацию ВПГ-1 в культуре клеток *Vero*, причем концентрация Z-изомера (*b*), подавляющая развитие вируса на 50% (IC_{50}), была близка к концентрации PMEA, известного антигерпетического препарата. Активность E-изомера (*c*) в аналогичных экспериментах была существенно ниже. Механизм действия соединений заключается в их фосфорилировании до дифосфатфосфонатов аденина, которые избирательно включаются в цепь вирусной ДНК и терминируют ее синтез. Оба изомера подавляли также резистентные к ацикловиру штаммы вируса, дефицитные по тимидинкиназе, поскольку для их активации не требуется первой стадии кинирования. Интересным свойством этих соединений была их способность подавлять репликацию вируса иммунодефицита человека в культуре клеток при концентрациях, близких к PMEA. Синтезированные дифосфаты соединений были субстратами как обратной траскриптазы ВИЧ, так и ДНК-полимеразы вируса герпеса, включались в 3′-конец праймер-матричного комплекса и терминировали дальнейшую элонгацию [21]. Полученные соединения являются одним из немногих описанных в литературе примеров, одновременно влияющих на развитие как ВИЧ, так и вируса герпеса.

Интересными оказались свойства *H*-фосфоната ацикловира. HpACV подавлял репликацию ВПГ в культуре клеток и понижал вероятность летального исхода для инфицированных ВПГ лабораторных животных [22]. Было отмечено, что подобно ацикловиру HpACV в комбинации с интерфероном α проявляет синергический эффект [23]. Характерной чертой HpACV было подавление ацикловиррезистентных штаммов вируса, дефицитных по тимидинкиназе, при этом его концентрация была всего в два

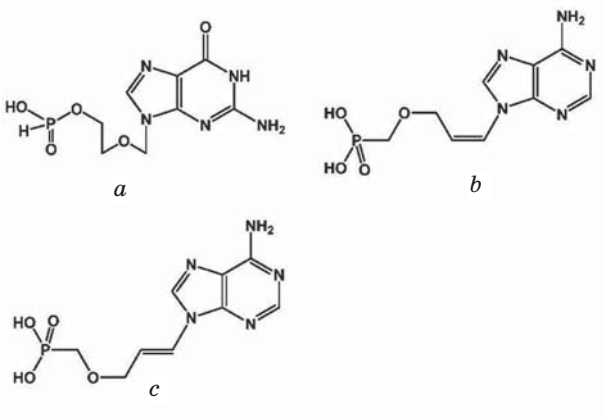


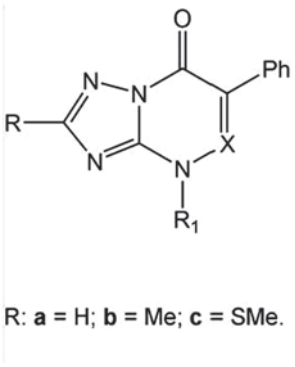
Рис. 3. **Фосфонатные производные нуклеозидов: HpACV (*a*), карбоциклический изостерный аналог гуанозина (*b*), (Z)-9-[3-(фосфонометоксипроп)-1-ен-1-ил] аденин (*c*)**

раза выше, чем в случае ацикловирчувствительных штаммов (в аналогичных экспериментах концентрация ацикловира, ингибирующая эти штаммы, увеличивалась почти в 500 раз) [24]. При этом появление резистентных к HpACV штаммов отмечалось через восемь пассажей, в то время как резистентность к ацикловиру была обнаружена через четыре пассажа, причем диапазон применяемых концентраций веществ был для HpACV 100 — 800 мкг/мл, а для ACD 2,5 — 100 мкг/мл. Эти результаты показывают, что резистентность к HpACV возникает существенно медленнее и при более высоких концентрациях по сравнению с ACV, что указывает на то, что, в отличие от ацикловира, HpACV ингибирует репликацию вируса без участия тимидинкиназы. При исследовании метаболизма HpACV на клетках *Vero* было показано, что после проникновения в клетку он в значительной степени непосредственно превращается в монофосфат ацикловира (ACVMP) и только небольшая его часть гидролизуется до ACV [10].

В нашей лаборатории был проведен сравнительный молекулярно-генетический анализ мутаций, возникающих при пассировании вируса в присутствии HpACV и ACV и приводящих к резистентным штаммам как к HpACV, так и к ACV, соответственно. Результаты этого анализа показали, что механизм, приводящий к подавлению вируса в присутствии ACV и HpACV, различен.

Производные триазинов

Совместно с Институтом органического синтеза им. Постовского (Екатеринбург) нами были изучены ациклические производные 1,2,4-триазоло[1,5-*a*]пиримидин-7-онов как ингибиторы репликации ВПГ-1 в культуре клеток *Vero* [25]. Структурные формулы соединений приведены на рис. 4.



№	X	R_1
1	CH	HO
2	N	HO O
3	N	HO
4	N	HO
5	N	HO

Рис. 4. **Структурные формулы производных 6-фенил-1,2,4-триазоло[1,5-a]пиримидина и (2-5) 6-фенил-1,2,4-триазоло[5,1-c] [1,2,4]триазин-7-она**

Было показано, что синтезированные соединения проявляют умеренную антигерпетическую активность на культуре клеток. Для изучения механизма действия были синтезированы трифосфаты этих соединений и показано, что они ингибируют ДНК-полимеразу вируса герпеса, причем их активность зависела от структуры соединения. Трифосфат соединения 3c (рис. 4), несущий метилтиогруппу, наиболее эффективно ингибировал встраивание радиоактивномеченого АМФ в 3′-конец праймерматричного комплекса. На основании полученных результатов можно предположить, что одной из мишеней действия соединений является герпесвирусная ДНК-полимераза. Мы пока не знаем, являются ли они ингибиторами нуклеозидного типа и связываются с активным центром энзима, или ненуклеозидного и связываются с энзимом вне активного центра. Испытанные соединения представляют собой новый тип ингибиторов вируса герпеса.

Молекулярно-генетический анализ клинических, резистентных к ацикловиру штаммов ВПГ

Как было отмечено выше, длительное применение лекарственных препаратов приводит к появлению резистентных штаммов вируса, что делает течение болезни неконтролируемым. Так, 4–7% изолятов ВПГ-1, полученных от иммунодефицитных пациентов, обладают устойчивостью к ACV. В 95% случаев устойчивость к ацикловиру обусловлена мутациями в гене тимидинкиназы и только в 5% случаев — мутациями в гене ДНК-полимеразы, но встречаются также вирусы, мутантные по обоим энзимам [26].

В генах тимидинкиназы и ДНК-полимеразы клинических изолятов и лабораторных штаммов, резистентных к ацикловиру и другим препаратам, выявлено большое количество мутаций, ряд которых приводит к потере активности энзима и возникновению резистентности к препаратам, активность которых зависит от тимидинкиназы вируса (ацикловир, пенцикловир, ганцикловир и другие нуклеозидные препараты).

На рис. 5 показано расположение мутаций в тимидинкиназе клинических изолятов вируса, резистентных к антигерпетическим препаратам.

Мутация R220H приводит к потере чувствительности вируса к ацикловиру [27], пенцикловиру и ганцикловиру, мишенью которых является вирусная тимидинкина-

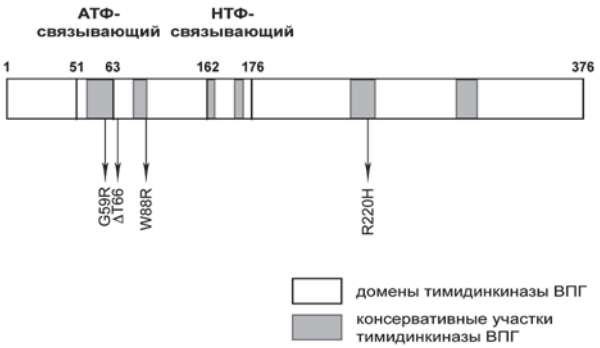


Рис. 5. **Расположение аминокислотных замен из разных клинических изолятов относительно консервативных участков и доменов тимидинкиназы ВПГ-1**

за. В работе [28] описана аналогичная мутация R220K в гене тимидинкиназы ВПГ-2, приводящая совместно с другими заменами к изменению субстратной специфичности энзима. Чувствительность резистентного к ацикловиру лабораторного штамма, несущего эту мутацию, к другим препаратам, например GCV и BVDU, также понижена на 1–2 порядка. В то же время соединение AraA, механизм действия которого не зависит от вирусной тимидинкиназы [23], подавляет репликацию этого штамма почти так же, как и штамма дикого типа. Мы показали, что эта мутация присутствует в гене тимидинкиназы лабораторного штамма, резистентного к HpACV. Однако она не влияет на активность HpACV, поскольку механизм его действия также не зависит от тимидинкиназы [27]. Мутация G59R находится в ATP-связывающем сайте энзима, важном элементе для функционирования энзима, поэтому мутация играет существенную роль в понижении его активности.

В ДНК-полимеразе ВПГ-1 было идентифицировано более 20 мутаций, ряд из которых существенен для функционирования энзима. Расположение мутаций в гене ДНК-полимеразы показано на рис. 6.

Замены I394K, P433S и V585M находятся в 3′-5′-экзонуклеазном домене энзима, причем мутация V585M располагается в консервативном участке ExoIII (572-585). В работе [29] показано, что мутации в этом участке влияют на чувствительность вируса к препаратам. Согласно литературным данным, мутация D581A, расположенная в непосредственной близости от V585M, приводит практически к полной потере 3′-5′-экзонуклеазной корректирующей активности энзима при частичном сохранении полимеразной активности [19]. Потеря или изменение корректирующей активности приводит к понижению точности синтеза ДНК-полимеразой и, следовательно, к повышению скорости возникновения мутаций.

Мутация N608S находится в консервативном участке C и способствует устойчивости лабораторного штамма ВПГ-1 к ACV и HpACV [24]. Было показано, что замена L702H в консервативном участке II ДНК полимеразы ВПГ-1 вызывает резистентность вируса к ацикловиру и сохранению чувствительности или незначительной резистентности по отношению к пенцикловиру и ганцикловиру. Замена гидрофобного лейцина 702 на пролин может приводить к изменению конформации b-листа в домене ладони, участвующего в координировании ионов магния и трифосфатного участка в активном центре энзима [30]. Аналогичный эффект вызывает мутация F716L, поскольку она располагается рядом с аминокислотным остатком D717, участвующим в координации ионов магния. При замене гидрофобного валина 715 на более полярный метионин происходит потеря чувствительности вируса к ацикловиру [31], а замена соседнего гидрофобного фенилаланина 716 на полярный лизин также оказывет сильное влияние на чувствительность энзима к этому препарату [27].

Согласно кристаллографическим данным [8], мутация M880T, расположенная достаточно близко от каталитической триады (D717, D886, D888) и участка связывания фосфатного остатка нуклеозидтрифосфата и ионов магния, может создать стерические препятствия для связывания остатков фосфата и обусловить понижение чувствительности вируса как к аналогам нуклеозидов, так и к PFA.

По данным литературы [32], замена N815S приводит к устойчивости штамма к ацикловиру и его аналогам. Согласно кристаллографическим данным (22), боковая

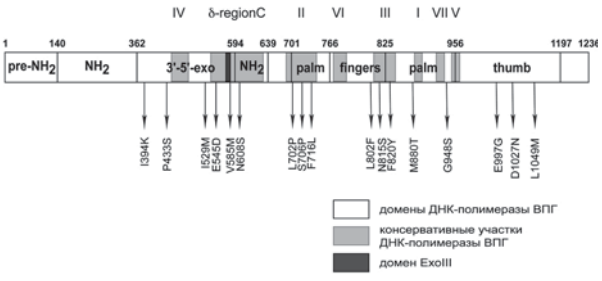


Рис. 6. **Расположение аминокислотных замен из разных клинических изолятов относительно консервативных участков и функциональных доменов ДНК-полимеразы ВПГ-1**

цепь N815 находится напротив основания нуклеотида, входящего в активный центр энзима. Компьютерное моделирование позволяет предположить, что боковая цепь мутантного остатка S815 имеет пространственную ориентацию, отличную от таковой остатка N815 исходного штамма. Мутантная по этому остатку полимераза не встраивает ACVMP в растущую цепь ДНК [33].

Мы проанализировали мутации в тимидинкиназе и ДНК-полимеразе четырех клинически изолированных штаммов вируса герпеса, резистентных к ацикловиру (Avd, Che, Tot, Sha), и показали, что большинство мутаций, обусловливающих резистентность, совпадают с уже описанными в литературе [27].

Однако в изоляте Che впервые были обнаружены аминокислотные замены в ДНК-полимеразе I159M и E545D, которые находятся в 3→5 экзонуклеазном домене и могут влиять на активность энзима. Большинство мутаций в ДНК-полимеразе, найденных в штамме Sha, также располагаются в экзонуклеазном центре, что обычно приводит к снижению корректирующей активности и обусловливает резистентность вируса к ACV. Таким образом, резистентность и клинических, и лабораторных штаммов объясняется мутациями как в ДНК-полимеразе, так и тимидинкиназе вируса герпеса, причем замены одного и того же аминокислотного остатка на другие аминокислотные остатки по-разному влияют на чувствительность энзимов к различным антигерпетическим препаратам

Данный обзор не претендует на полное изложение всего объема материала по поиску антигерпетических препаратов. После открытия ацикловира прошло 30 лет, за это время появилось много эффективных препаратов, достигнуто существенное понимание стратегии поиска препаратов, один из результатов которого — создание депо-форм антигерпетических препаратов. Было опубликовано много обзоров и статей с подробным описанием экспериментальных и клинических исследований (см., например, [33]). Нам хотелось только показать, что изучение вируса герпеса и поиск ингибиторов остается доныне важной проблемой и требует совместных усилий химиков, биологов, фармацевтов.

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ПОШУК ІНГІБІТОРІВ РЕПЛІКАЦІЇ ВІРУСУ ГЕРПЕСУ: 30 РОКІВ ПІСЛЯ АЦИКЛОВІРУ

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В огляді здійснено аналіз вивчення та застосування різних хімічних сполук як інгібіторів реплікації вірусу герпесу. Проте він не претендує на повний виклад усіх досліджень пошуку активних антигерпетичних препаратів. З часу відкриття першого антигерпетичного препарату — ацикловіру — минуло 30 років. За цей час з'явилося багато активних хімічних сполук, які стали основою для створення антивірусних препаратів, прийшло істотне розуміння стратегії пошуку препаратів, результатом якого, зокрема, було створення депо-форм антигерпетичних препаратів. На підставі великого обсягу опублікованого експериментального матеріалу автори роблять висновок про те, що вивчення вірусу герпесу і пошук інгібіторів його реплікації залишається й досі важливою проблемою і потребує спільних зусиль хіміків, біологів та фармацевтів.

Ключові слова: вірус герпесу, противірусні препарати, ацикловір.

SEARCH OF INHIBITORS OF HERPES VIRAL REPLICATION: 30 YEARS AFTER ACYCLOVIR

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Analysis of study and using of different chemical compounds as inhibitors of herpes virus replication is given in the review. However, it does not apply for full details of all the studies on active antiherpetic drugs finding. It's been over 30 years since the discovery of the first antiherpetic drugs — acyclovir. Meanwhile, lots of active chemical compounds appeared that have been brought to the antiviral drugs. An essential understanding of strategies for finding drugs came in, one of which was establishment of depot forms of antiherpetic drugs. On the basis of the vast published experimental material the authors concluded that the study of the herpes virus and search for inhibitors of its replication is still an important issue and requires the efforts of chemists, biologists, pharmacists.

Key words: herpes virus, antiviral drugs acyclovir.

UDK 543.645.6:665.123

RECOMBINANT HORSERADISH PEROXIDASE FOR ANALYTICAL APPLICATIONS

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The article deals with prospects of using recombinant horseradish peroxidase in analytical biochemistry and biotechnology. Problems of recombinant horseradish peroxidase cloning in different expression systems, possible approaches to their solution, advantages of recombinant recombinant horseradish peroxidase and recombinant horseradish peroxidase-fusion proteins for immunoassays are considered. Possibility for development of mediatorless bienzyme biosensor for peroxide and metabolites, yielding hydrogen peroxide during their transformations, based on co-adsorption of recombinant horseradish peroxidase and the appropriate oxidase was demonstrated. The possibility to produce a fully active recombinant conjugate of recombinant horseradish peroxidase with human heart-type fatty acid binding protein, which may be used in competitive immunoassay for clinical diagnosis of acute myocardial infarction, and recombinant conjugates (N- and C-terminus) of recombinant horseradish peroxidase with Fab-fragments of the antibody against atrazine, which may be applied for atrazine pesticides detection, are demonstrated for the first time.

Key words: recombinant horseradish peroxidase, fusion proteins, immunoassays, mediatorless bienzyme biosensor, Fab-fragments of antibody, heart-type fatty acid-binding protein, atrazine pesticides.

Horseradish peroxidase isoenzyme C (HRP, EC 1.11.1.7), a heme- and Ca^{2+}-containing glycoprotein, is a member of the superfamily of plant peroxidases [1–3] that are able to utilize hydrogen peroxide to catalyze the one electron oxidation of a wide variety of organic and inorganic substrates. Whereas plant peroxidases find interest for applications in various biotechnological processes, like bleaching and degradation of organic compounds (e.g. phenols and lignin), HRP specifically is used in analytical biochemistry and biotechnology as a marker enzyme for antibodies, DNA and low molecular mass analytes. Broad substrate specificity and high catalytic activity and stability determined the world-wide application of HRP in bio- and immunosensors, chemiluminescent, fluorescent and electrochemical detection systems, DNA microarrays and biochips with HRP-based colorimetric detection [4–6, 14, 56].

Progress made in peroxidase gene heterologous expression opened up the prospect to study structure-function relationships by means of genetic engineering. The baculovirus expression system allowed production of recombinant HRP in soluble, active and glycosylated form [7], however, this system is laborious and not as wide spread as expression in *E. coli*. Expression of HRP in yeast indicated only very low expression of active enzyme [8].

Up to date for the production of wild-type as well as mutant recombinant HRP the *E. coli* expression system is mostly used [9, 10]. Recombinant HRP forms inclusion bodies containing only traces of heme if expressed in bacterial cytoplasm. Multistep refolding and reactivation of recombinant apo-peroxidase with the prosthetic heme group is complex as the protein contains four disulfide bonds and in addition must bind two Ca^{2+}-ions per molecule. Moreover, the plant-derived enzyme contains 18% carbohydrates via 8 glycosylation sites. Crystal structure of recombinant HRP has been solved, revealing the presence of two domains formed by a total of ten α-helices [11].

Among of the factors leading to the formation of inclusion bodies upon expression of

cysteine-rich proteins is the reduction potential of *E. coli* cytoplasm, preventing correct formation of disulfide bonds [12]. One solution to this problem was fusion of such a protein to a signal peptide for secretion into the bacterial periplasm. After translocation the signal peptide is cleaved off and a correctly folded protein with disulfide bonds can be formed under the oxidizing conditions of this compartment. This approach has been successfully applied for the production of soluble active recombinant HRP, though with rather low yield [13].

In biotechnological applications, however, a more effective and reliable way for production of recombinant HRP is desirable. Introduction of a C-terminal His-tag facilitated renaturation and purification of recombinant peroxidase from inclusion bodies. Moreover, by addition of a His-tag to the recombinant peroxidase it was envisaged to facilitate downstream processing, in particular of the dilute solutions obtained after the refolding procedure. The high yield and high specific activity obtained with the optimized protocol enables to produce sufficient recombinant enzyme for the development of biosensors in which electrons are directly transferred from the electrode to the immobilized peroxidase [4, 6] or in which the sensitivity of the enhanced chemiluminescence reaction is increased [14]. In particular, His-tag recombinant HRP co-adsorbed with corresponding oxidases producing H_2O_2 can be considered as promising for future multienzyme biosensor development [4, 5].

Principal possibility of the development of a mediatorless bienzyme biosensor for peroxide and metabolites, yielding hydrogen peroxide during their transformations, based on co-adsorption and cooperation of HRPhis, capable of efficient direct electron transfer, and the appropiate oxidase, e.g., LysOx, was demonstrated. Amperometric bienzyme biosensor for the determination of L-lysine based on LysOx and HRP containing 6-His tag at the C-terminus physically co-immobilized on the surface of polycrystalline gold electrodes is shown to be simple in manufacturing and operation, sufficiently effective and highly reproducible. Efficient direct electron transfer between gold electrodes and immobilized His-tag HRP makes it possible a mediatorless detection of hydrogen peroxide that is released during the enzymatic oxidation of L-lysine, thus decreasing the number of components in the system used for the detection [4–6].

Genetic engineering approach offers new opportunities for broad application of recombinant HRP to design highlysensitive immunobiosensors of a new generation, based on the recombinant DNA technology.

Survey on recombinant conjugates for analytical application

Horseradish peroxidase is a key marker enzyme for immunodiagnostics. Enzyme immunoassays for the detection and quantitative analysis of various substances are based on coupling of marker enzymes like HRP with antigens or antibodies. However, all the chemical conjugation methods result in partial inactivation of the enzyme and heterogeneity of the conjugates, which in turn influence specificity and sensitivity of the assays. With the advance of genetic engineering it became clear that genetic in frame fusions of antigens/antibodies and enzymes would provide many of the desirable features of conjugates for use in immunoassays, in particular homogeneity, 1:1 stoichiometry, reproducibility and ease of production [15].

Early fusion proteins contained the bacterial enzymes β-galactosidase [16–18] or alkaline phosphatase [19–21], which can be easily expressed in *E. coli*. In addition to these enzymes, bioluminescent or fluorescent marker proteins such as aequorin or green fluorescent protein [22, 23] have been used as fusion partners for a model octapeptide. Genetic fusion was also employed to construct conjugates with protein A [24] or an in vitro biotinylated polypeptide tag for β-galactosidase [25]. The genetic approach is particularly attractive for fusions to small peptides with numerous functional groups, which are difficult to control [26], or with human proteins which often are not easily available [27]. Whereas *β*-galactosidase is solubly expressed in the cytosplasm, the disulfide-containing alkaline phosphatase is secreted into the periplasm, thus also broadening the spectrum of fusions to disulfide-containing proteins [28, 29]. The drawback of the lower specific activity of the bacterial alkaline phosphatase in comparison to the calf intestinal alkaline phosphatase routinely used in chemical conjugations could be partly overcome by using a genetically engineered mutant of the bacterial enzyme with increased activity [30]. Recombinant conjugates of antibodies with alkaline phosphatase [31–35], luciferase [36], and peroxidase *Arthromyces ramosus* [37] were obtained earlier.

A principal problem associated with β-galactosidase or alkaline phosphatase fusions is their tetrameric and dimeric structure, respectively, which likely leads to an increased apparent affinity (avidity) of a conjugate in comparison to the free antigen. This is not desirable for the development of competitive immunoassays. On the other hand, horseradish peroxidase, which is very popular for preparation of enzyme-conjugates [38], can only be expressed in *E. coli* in the form of inclusion bodies. The yield of refolded and reconstituted (with heme) recombinant peroxidase used to be rather low [9, 10], which has so far precluded the use of this enzyme in the genetic fusion approach. Progress in heterologous expression in *E. coli* and reactivation of recombinant HRP carrying a C-terminal oligohistidine tag (HRPhis) [13] opened the prospect of producing a recombinant conjugate of HRPhis with a marker enzyme for application in immunoassays.

Recombinant conjugates of HRP with protein antigens

Earlier we've exemplified that a fusion protein of peroxidase and human heart fatty acid binding protein (H-FABP) can be used as a recombinant tracer in immunoassays for detection of H-FABP [39]. This small (15 *kDa*) cytosolic protein is a member of a protein family specialized in transport of fatty acids and is highly abundant in heart muscle (0.52 mg/g heart tissue). Its rapid release into the circulation and its good tissue specificity make it an ideal early marker for clinical diagnosis of acute myocardial infarction [27, 40].

A fusion protein comprising HRP and human heart-type fatty acid-binding protein (H-FABP) was constructed by introducing the coding part of the human H-FABP cDNA into the *Xma*III site of the expression vector for horseradish peroxidase, *pETHRPhis* [13], just in front of the 6xHis tag (Fig. 1).

Recombinant HRP-FABPhis conjugate has the same Soret band absorption with a maximum at 403 nm as native peroxidase and recombinant HRPhis, indicating that the Fe^{3+} co-ordination by heme as well as proximal and distal histidines is not affected. These data indicate that C-terminal extension of the recombinant peroxidase with the 15 kDa human H-FABP has no drastic influence on the activity of the recombinant conjugate, which values corresponds to that for recombinant HRPhis alone and the plant-derived enzyme (1000 U/mg against ABTS substrate)

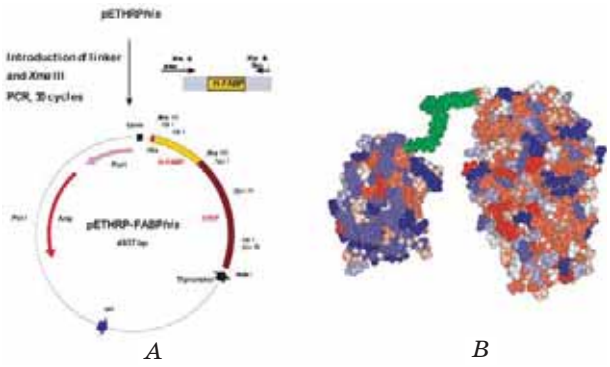

Fig. 1. **Cloning scheme (*A*) and spatial model (*B*) of HRP-FABP conjugate**

[13]. At the same time the recombinant conjugate bound fatty acids as shown qualitatively by a gel elution assay with radioactive oleic acid (data not shown) and was recognized by a sandwich ELISA with two monoclonal antibodies [27] indicating the structural integrity of the FABP part.

The competitive assay format with its reduced number of incubations can be successfully employed to develop fast immunoassays. Solid phase immunoassay is based on competition of free H-FABP with HRP-FABP conjugate for binding sites on microtiter plates coated with polyclonal antibodies against H-FABP. A conjugate of plant peroxidase and human H-FABP has been synthesized based on periodate oxidation of oligosaccharides [41].

The typical calibration curves shown in Fig. 2 demonstrate the wide measuring range (3 orders of magnitude of H-FABP concentration) and the good detection limit — 1,5 ng H-FABP per ml of sample (0.05 ng/well). The intraassay variation coefficient was between 4 and 8% [39]. The detection limit of this new immunoassay is similar to that observed with sandwich-type ELISAs using monoclonal antibodies against human H-FABP (0.02–0.1 ng/well) [27, 40]. Interestingly, at higher concentrations H-FABP competed better with the recombinant conjugate than with the chemically prepared conjugate for binding to the polyclonal capture antibodies. One explanation could be the presence of chemically prepared conjugate consisting of HRP with more than one H-FABP attached, which would exhibit a higher apparent affinity due to multipoint binding to the immobilized antibodies.

To challenge the competitive ELISA we analyzed a set of plasma samples periodically withdrawn from one patient with diagnosis of AMI over a period of 24 h after admission to the hospital (Fig. 3) [39]. The values for

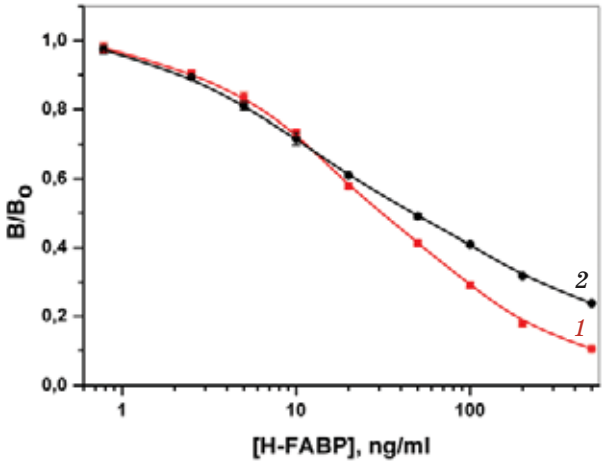

Fig. 2. **Calibration curves for the competitive immunoassay. Competition of *1* — recombinant (-●-) or *2* — chemically prepared conjugate (-■-) with H-FABP**

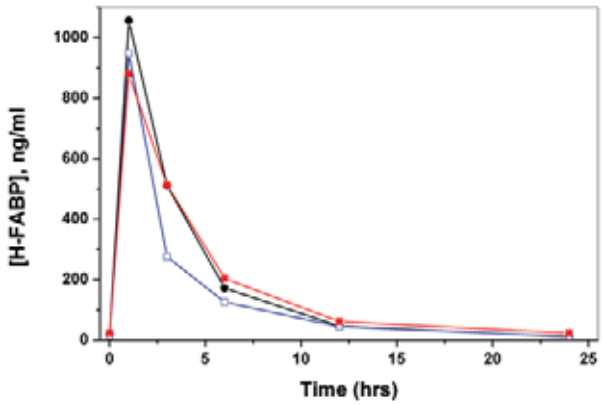

Fig. 3. **Comparison of FABP plasma concentration, obtained with different immunoassay formats. Profile of patient No. 14: -●-sandwich ELISA, -□- electrochemical immunosensor, -■- competitve ELISA**

H-FABP concentrations in the plasma samples assayed with the competitive ELISA exhibited good correlation with those obtained by the reference sandwich ELISA and the developed EUROCARDI immunosensor [27, 40].

Thus we have opened up for the first time the possibility to reproducibly produce a recombinant conjugate of a protein antigen with horseradish peroxidase as marker enzyme for use as tracer in competitive immunoassays [39]. The applicability of this genetically engineered fusion protein with a defined 1:1 stoichiometry for a clinically relevant analyte, the human heart fatty acid binding protein, has been shown with plasma from a patient after myocardial infarction. Our approach paves the way for broad application of the popular peroxidase marker enzyme in competitive immunoassays employing genetically engineered conjugates. We have already extended the concept by preparation of a recombinant conjugate of peroxidase and human myoglobin, another analyte important for early detection of myocardial infarction.

Recombinant conjugates of HRP with (Fab) antibodies fragments

The functional expression of the recombinant conjugate of HRP and antibody fragments in *E. coli* is associated with a number of difficulties, since there is no post-translational glycosylation of proteins in *E. coli* cells, resulting in low solubility and aggregation of the resulting protein. This problem can be solved by replacing the expression system. For instance, it has been shown that methylotrophic yeast *Pichia pastoris* is a more suitable medium for antibody expression than *E. coli* cells [42, 43].

HRP [44] and antibody fragments [45] were successfully expressed individually in *P. pastoris* cells, both in the single-stranded form scFv [46, 47] and in a Fab form [48]. Moreover, certain immune conjugates have also been created using this expression system [49–51]. It has been demonstrated that gene expression in the *Pichia pastoris* system in the secreted form considerably simplifies the scaling of the process for biochemical applications [52].

Progress in functional secreted expression of HRP and antibodies in *Pichia pastoris* [44, 53] open the prospect to produce recombinant conjugates of *HRP* with antibodies for application in immunoassays. However, the production of recombinant conjugates is an appreciably complicated task, since it remains thus far impossible to reliably predict the structure of the desired conjugate; hence, loss of the functional activity of both the marker enzyme and antigen is possible, due to the incorrect folding of two components.

General versatile expression system for recombinant conjugates of peroxidase with *Fab* fragments of antibodies production has been elaborated based on *pPICZalfa* vector and *X33 P.pastoris* strain (*Invitrogen*). These systems provide secreted, methanol-inducible expression in cultural medium two types of conjugates where the peroxidase part genetically fused to *N*- or *C*-terminal part of variable heavy chain of antibody via short flexible linker sequences (Gly_4Ser) (Fig. 4) [54].

To exemplify the applicability of this approach for the first time we have produced set of conjugates of peroxidase with *Fab* against atrazine pesticides (Fig. 4).

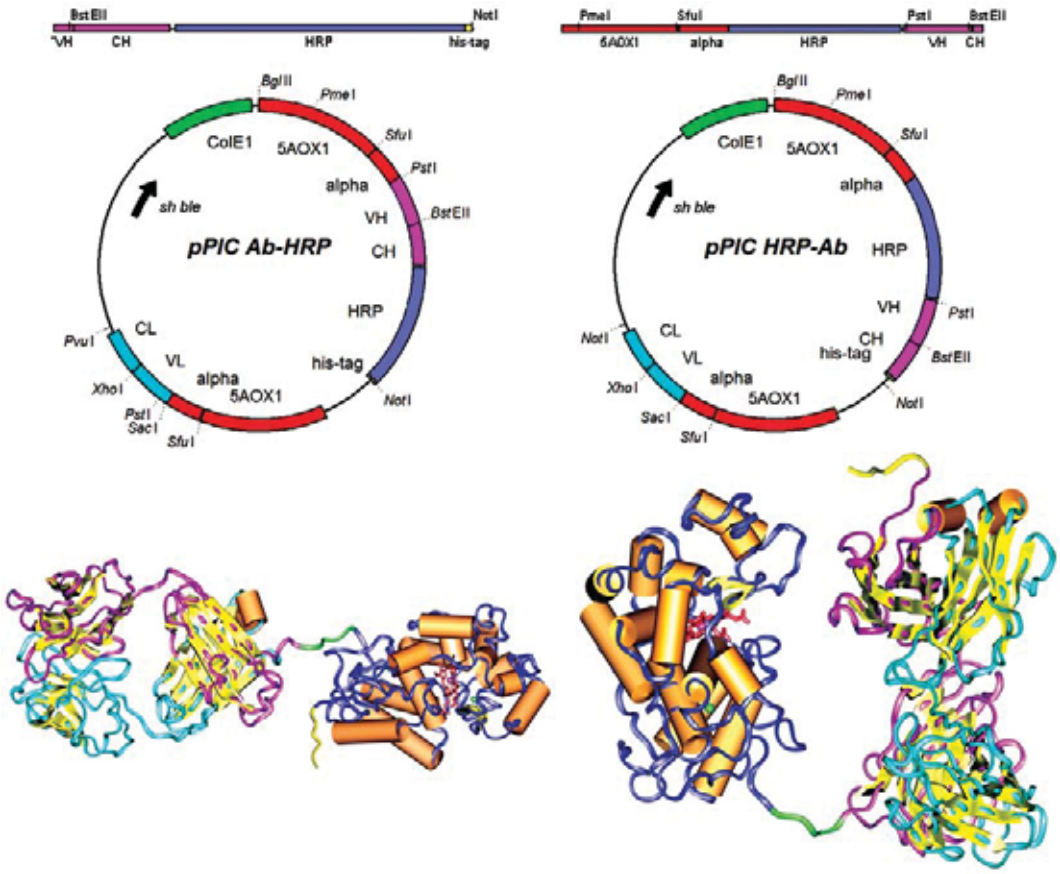

Fig. 4. **Cloning schemes and spatial models of recombinant conjugates: Fab-HRP and HRP-Fab (left and right panel respectively)**

The developed expression vectors allow simple recloning of any variable heavy (*PstI*/*BstEII* sites) and light (*BamHI*/*XhoI* sites) chains, thus providing general vectors for recombinant conjugates of peroxidase with antibodies production in *P. pastoris*.

The total yield of recombinant conjugates was approximately 3–10 mg per 1 L of the *P. pastoris* culture supernatant. A relatively low yield of secreted conjugates correlates with the yield upon expression of the HRP gene only [54]. We believe that one of the factors that have a negative effect on the yield of the secreted product is the excessive glycosylation of the peroxidase component of the conjugate, which is typical of *P. pastoris* cells. In order to verify this hypothesis, it may be reasonable to remove all N-glycosylation sites in HRP or replace HRP with another reporter protein, such as EGFP.

In order to confirm the antigen-binding activity of recombinant conjugates, we selected the scheme of indirect competitive single-stage ELISA carried out on the wells with an immobilized atrazine–BSA conjugate. The data obtained attest to the presence of both catalytic and antibody activity in all forms. However, the low activity of the HRP-Fab in comparison with the C-terminal conjugate Fab-HRP may attest to the fact that the mutual spatial arrangement of two components of the chimeric protein in this case results in a decrease in the catalytic activity of peroxidase. Typical calibration curve (Fig. 5) allows one to determine the atrazine concentration over a wide range, from 0.1 to 50 ng/ml; the variation coefficient being no higher than 8%. IC_{50} is equal to 3 ng/ml, which agrees well with the results of atrazine determination by a two-stage ELISA procedure using recombinant Fab fragments of the same antibody K411B [54] and with the data on the single-stranded mini-antibody (scFv) obtained earlier in *E. coli* [53, 55]. Thus, the recombinant conjugates of peroxidase with Fab fragments of antibody against atrazine obtained in the present study possess functional activity and can be used to determine atrazine via ELISA.

We have for the first time demonstrated the possibility to produce a fully active recombinant conjugate of a protein antigen with horseradish peroxidase as marker enzyme for

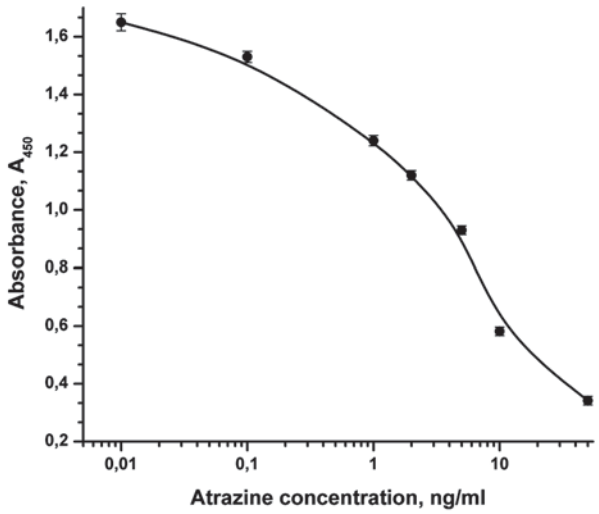

Fig. 5. **Calibration curve for atrazine determination in competitive ELISA with recombinant conjugate of Fab-HRP**

use as tracer in competitive immunoassay for a clinically relevant analyte. We have already extended the concept by preparation of a recombinant conjugate of peroxidase and human myoglobin, another analyte important for early detection of myocardial infarction.

The possibility of using a recombinant, functionally active HRP (as a marker enzyme) conjugated with Fab fragments of the antibody against atrazine was shown for the first time. Recombinant conjugates were obtained in which the Fab fragment of an antibody is bound both to the N- and the C- terminus of peroxidase. Both these variants manifest immunological and catalytic activity.

Thus successful genetic engineering towards horseradish peroxidase opens the new opportunities of using this traditional marker enzyme for analytical and biomedical application.

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ВИКОРИСТАННЯ РЕКОМБІНАНТНОЇ ПЕРОКСИДАЗИ ХРОНУ ДЛЯ АНАЛІТИЧНИХ МЕТОДІВ

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Статтю присвячено перспективам застосування рекомбінантної пероксидази хрону в аналітичній біохімії та біотехнології. Розглянуто проблеми клонування пероксидази хрону в різних експресійних системах, можливі підходи до їх вирішення, а також переваги використання в імуноаналізі рекомбінантної пероксидази хрону і злитих протеїнів на її основі. Показано принципову можливість створення безмедіаторного біензимного біосенсора для виявлення пероксиду водню і метаболітів, що утворюють його під час трансформації, на основі коадсорбованих рекомбінантної пероксидази хрону та відповідної оксидази. Уперше показано можливість одержання функціонально активного рекомбінантного кон'югату пероксидази хрону із серцевим протеїном людини, що зв'язує жирні кислоти, який може бути застосовано в конкурентному імуноаналізі для діагностики інфаркту міокарда, а також N- і C-кінцевих рекомбінантних кон'югатів пероксидази хрону із Fab-фрагментами антитіл проти атразину — для виявлення пестициду атразину.

Ключові слова: рекомбінантна пероксидаза хрону, злиті протеїни, імуноаналіз, безмедіаторний біензимний біосенсор, Fab-фрагменти антитіл, серцевий протеїн, що зв'язує жирні кислоти, пестицид атразин.

ИСПОЛЬЗОВАНИЕ РЕКОМБИНАНТНОЙ ПЕРОКСИДАЗЫ ХРЕНА ДЛЯ АНАЛИТИЧЕСКИХ МЕТОДОВ

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Статья посвящена перспективам применения рекомбинантной пероксидазы хрена в аналитической биохимии и биотехнологии. Обсуждаются проблемы клонирования пероксидазы хрена в различных экспрессионных системах, возможные подходы к их решению, а также преимущества использования в иммуноанализе рекомбинантной пероксидазы хрена и слитых протеинов на ее основе. Показана принципиальная возможность создания безмедиаторного биэнзимного биосенсора для выявления пероксида водорода и метаболитов, образующих его при трансформации, на основе коадсорбированных рекомбинантной пероксидазы хрена и соответствующей оксидазы. Впервые показана возможность получения функционально активного рекомбинантного конъюгата пероксидазы хрена с сердечным протеином человека, связывающим жирные кислоты, который может быть применен в конкурентном иммуноанализе для диагностики инфаркта миокарда, а также N- и C-концевых рекомбинантных конъюгатов пероксидазы хрена с Fab-фрагментами антител против атразина — для выявления пестицида атразина.

Ключевые слова: рекомбинантная пероксидаза хрена, слитые протеины, иммуноанализ, безмедиаторный биэнзимный биосенсор, Fab-фрагменты антител, сердечный протеин, связывающий жирные кислоты, пестицид атразин.

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MODERN TECHNIQUES OF IMMUNOCHEMICAL ANALYSIS: INTEGRATION OF SENSITIVITY AND RAPIDITY

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The review covers history and development prospects of immunochemical analysis. Advantages and prospects of antibodies as detecting agent, modern requirements to immune-analytical methods and preconditions for two clusters formation (homogeneous relatively insensitive rapid assays and heterogeneous high sensitive and long duration assays), as well as the ways of improvement of analytical characteristics of these immunoassays are considered in detail. Forecast regarding most promising directions of immunochemical analysis, in particular, multiparametric analytical systems is made. Possibilities to develop universal immune-analytical systems, comprising high sensitivity of heterogeneous assays and detection rapidness of homogeneous assays (for example, immunoassays using polyelectrolytes or magnetic colloidal particles) are considered.

Key words: immunochemical analysis, homo- and heterogeneous immunoassays.

Potential and advantages of antibodies as bioanalytical receptors

In course of accumulation of knowledge about antigen-antibody interaction as highly affine and highly specific reaction an interest to antibodies as the means for the detection of antigens of various chemical nature was growing. The history of immunoassay started from immune precipitating methods, in which after extended incubations visually detectable insoluble antigen-antibody aggregates were formed. Important is the possibility to carry out the immunoprecipitation with the use of unfractionated antiserums, containing antibodies to a defining compound, and various samples without their pre-processing (except for very turbid mediums). Such procedural simplicity, the initial reason of which was the absence of tools for more sensitive detection of immune complex, provided high viability of this approach, applied till the moment for the assay of many proteins and determination of blood-group specificity [1]. Maximal reduction of the determination duration was not demanded from immune precipitation; obtaining of the results of clinical test in 1–2 days after sampling was perceived as a norm several decades ago. More continuing incubation was not observed as a disadvantage of the procedure, but as a definite guarantee of greater reliability and reproducibility of the results due to achievement of final (equilibrium) stage by the immune-precipitating processes.

An important, revolutionary progress within the development of immunoassay was the occurrence of analytical systems, in which one of implemented immune reagents was the complex with a marker, detected in extremely low concentrations. In the beginning of 1950-s such procedure was fulfilled for radio-active isotopic tags by Yalow and Berson [2], who were awarded for this development in 1977 with the Nobel Prize. A bit later the methods started developing with the use as the tags of enzymes, fluorophors, other compounds. New systems of detection were actively described, allowing to detect various compounds in the concentrations up to 10^{-10}–10^{-12} M in several hours. Transfer to non-isotopic tags excluded the need in special equipment and safety measures and thus called even more wide spread of immune-diagnostics. The main area of its implementation was and remains to be medicine, which greatly exceeds in the scopes of commercialization the ecological monitoring, control of quality and safety of food products and agricultural raw materials, other areas, in which immunoassay is also actively used.

In recent years many researchers has suggested to use in the analysis other receptor molecules as an alternative to antibodies. On the one hand, these are natural agents with similar recombination of structural elements of a molecule and possibility of obtaining of millions of its variants with different specificity [3–5]. On the other hand — synthetic compounds, in which a mark of an analyte is created artificially, providing the possibility of its selective detection [6] [7–10]. Nevertheless, antibodies remain to be and in the near future, probably shall remain to be the receptor, the most widely implemented for specific detection and quantification of content of the compounds of various nature [11]. To a large extent it is conditioned with a significant «allowance», which was obtained by antibodies as a result of their active use for decades. At the moment there are commercially available antibodies to dozens of thousands compounds of practical importance. The approaches were developed, which allow obtaining antibodies to the agents, which traditionally were not observed as inducers of immune response — ions of heavy metals [12–14], carbon nanoparticles [15, 16], vitamins [17], highly toxic compounds [18]. The technologies of obtaining of hybrids and display libraries allow to perform rapid screening and selection of antibodies with necessary specificity, and then producing the given antibodies in unlimited quantities [19, 20]. Affinity and specificity of antibodies can be purposefully changed with the use of methods of gene engineering, which lately have been intensively implemented for molecular design of antigen-binding sites of antibodies [21, 22]. The procedures of conjugation of antibodies with various markers were developed, combining high product output and high level of preservation of its functional properties [23].

Modern requirements to immune-analytical methods

However, although we may give a unique reply on the question of choosing of bio-receptor element, the situation with the choice of an analytical method is much more complicated. Now, as the labels in immunoassay (in commercial tests and in the developments recommended to the introduction into the practice) are used enzymes, fluorophores, liposomes, co-factors, various nanoparticles, etc. The analysis is performed in the volume of a solution, at the surface of polystyrene plates, electrodes, in flow-through cells, membrane pores, etc. Formation of immune complexes is registered by photometric, fluorometric, amperometric, potentiometric, gravimetric, magnetic detectors, etc. By this each of these «etc.» — are the dozens of more rare variants, representing nevertheless, the interest for the researchers.

What is the ground for such variety and is it needed? Should we choose one, the best at the moment method, and use exactly it for the determination of various antigens and for the solution of various practical tasks? As such solution shall simplify demands to equipment and reagents, skills of specialists, interpretation of obtained outcomes. Unfortunately or luckily, but such unification appears to be possible. Immune-chemical methods are used for solution of various tasks, differing in practical requirements. On the one hand, these are tests, performed in specialized, stationary laboratories, from which it is expected the maximum sensitivity and reliability, but is pretty acceptable the range of several hours between sampling and obtaining of the assay results. On the other hand, in a significant number of cases it is required rapid, for minutes, obtaining of the information directly at the site of sampling (at the site of the primary screening medical examination, by attending of a doctor of the patient in house, in case of self-control of the patient, at examination of natural and industrial objects). These two niches of immunoassay at the moment are successfully filled with various analytical methods.

The task of reliable highly sensitive diagnostics is solved by enzyme-linked immunosorbent assay, immune-fluorescent assay, radio-immune assay — heterogeneous methods, based on continuing (dozens of minutes or hours) incubations of reagents till achievement by immunochemical reaction of equilibrium condition, separation of bound and non-bound components and high-sensitive quantitative registration of corresponding markers in the set of immune complexes.

An alternative approach is performance of homogenous immunochemical reactions, achieving equilibrium in minutes, and direct detection on this or that parameters of content in a reaction medium of created immune complexes. To this group we should assign immune-analytical systems, based on modulations of activities of an enzyme marker [24], changing of polarization of a fluorescent marker [25], registration of immunochemical complexes on changing of optical properties of the medium (nephelometry) [26], etc. Due to the absence of the stage of reagents separation

and corresponding influence on registered signal of components of samples (so named matrix effect) and non-reacted immunoreagents, and as well as due to relatively low sensitivity (in comparison with the registration of markers on heterogeneous immunoassays) the given analytical methods are generally characterized by sufficiently higher limits of detection. Initially they were also developed as the methods for stationary laboratories, but the development of technologies and miniaturization of the blocks of data processing allowed suggesting the number of portable mobile systems, suitable for field tests (first of all — for the registration of polarization of fluorescence) [27].

To the test-systems for rapid screening with low sensitivity it is worth to assign also membrane immunochromatographic test-systems (test-strips) [28–30], in which rapidness of detection is assured by the use of homogenous (in the volume of flowing on test-strip fluid) and rapid heterogeneous (in micro-volumes of membrane pores) interactions, and immunoreagents are conjugated with coloured ultradisperse markers (colloidal gold, latexes, etc.) for rapid direct detection [31, 32].

So, the modern situation in immune diagnostics is characterized by some established «division of duties» between relatively long (hours) analytical methods with low limit of detection and rapid (minutes) methods, defining sufficiently higher concentrations of target compound, and in the number of cases — representing only qualitative information that its content in the sample is higher than a definite limit. Each of these classes of methods is well adapted for its special tasks, and its practical use is supported by developed technologies on production of corresponding test-systems, presence of serial equipment for assaying and final measurements, informing and professional training of specialists — users of the developed test-systems.

Nevertheless, the established situation should not be considered as a final optimal solution. The wish to combine the benefits of the given approaches and to suggest immune-analytical systems, comprising high sensitivity and rapidness of detection is natural. In the present review we shall observe the developments held in this area in recent years. The figure 1 summarizes the existing variety of approaches, targeted at improvement of the characteristics of immunoanalytical systems.

Methods of reduction of detection limit in immunoassays

Achievement of a low limit of detection of immunoassay demands the combination of high-affine interaction of antibodies with corresponding antigens and the potential of marker detection (or a parameter, accompa-

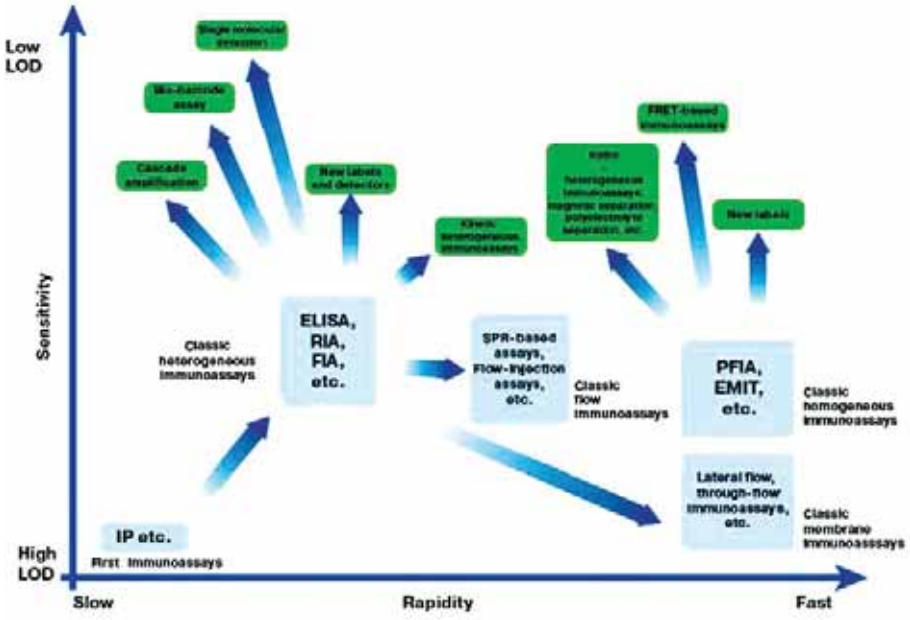

Fig. 1. **Variety and evolution of immunoassays.**
Abbreviations: IP — immunoprecipitation,
ELISA — enzyme linked immunosorbent assay, RIA — radioimmunoassay, FIA — fluorescent immunoassay,
PFIA — polarization fluorescence immunoassay, EMIT — enzyme multiplied immunotechnique,
SPR — surface Plasmon resonance

nying formation of the immune complex) in extremely low concentrations.

In 1986 Jackson and Ekins [33] held theoretic comparison of non-competitive and competitive immunoassays in the notions of sensitivity, precision, kinetics and working range of analyte. They defined theoretic limits of detection of these methods, their connection with the characteristics of antibodies.

It's worth to note, that the peculiarity of dependence between the binding constant of immunochemical reaction and the limit of detection of the target antigen significantly depends on the accomplished format of the immunoassay — non-competitive or competitive. In the first case the antigen is detected directly in the process of immune complex formation, the way it is happening, for example, in a sandwich format of the analysis with formation of complexes antibody — antigen — labelled antigen. In the second case a competition between the antigen in a sample and the second antigenic agent for binding with antibodies is registered. For non-competitive analysis it is potentially possible to detect extremely small concentrations of antigen, if it is allowed by the sensitivity of marker detection or direct detection of immune complex, as well as low background signal. There is a number of works, describing the possibility to detect a single antigen molecule with these sources of signal strengthening [34, 35].

A significant role is played by the optimization of the ratio of reagents used. It should assure, on the one hand, a minimal limit of detection, and on the other hand — maximum reliability and (for quantitative methods) precision of analysis. These requirements cannot be performed simultaneously and demand definite compromise solutions. For the formats of analyses with direct dependence between the concentration of antigen and the number of detected markers (such as a sandwich scheme) increase of concentration of immune reagents enhances the number of detected complexes. However this increase may be accompanied by the growth of non-specific (background) binding of markers. For competitive analysis with reverse dependence between the concentration of analyte and the number of detected markers the reduction of the limit of detection is assured by low concentrations of immunoreagents [36], but it is accompanied by low amplitude of signal and low precision of determination [37].

Despite doubtless importance of affinity of immune interaction for high sensitive assay, it should not be suggested that its growth to infinity shall assure unlimited lowering the detection limit. In this case the issue about minimal detected concentration of a marker becomes critical. Nevertheless, for modern practice the choice of the most affine antibodies from the variety of available ones allows reducing significantly the detection limit, achieving in the number of cases subnanogram levels [38].

Traditionally immunologists note the existence of natural limits on affinity of complex-formation of antibodies with antigens, defined by the nature of induction of immune response. This is connected with the fact that for antibodies with the kinetic constant of dissociation of the order 10^{-4} sec^{-1} and less the time of half-life of the complex of antigen with B-cell receptor becomes greater, than the time of endocytosis of this complex. Respectfully, further increase of the time of half-life of the complex already does not assist B-cell proliferation [39]. Maximum value of the kinetic constant of association is defined by the speed of diffusion of immunoreagents in the solution. In this respect for the antibodies of IgG class, specific to protein antigens, the equilibrium constant of association usually do not exceed 10^{10} M^{-1} [40]. However, these limitations do not exclude the possibility of that, a significantly higher level of complementarity with antigen-binding site of antibodies, and correspondently, a higher constant value of binding is achieved for some antigens. There is also a number of works describing non-dissociating complexes of antigen-antibody with infinite affinity [9, 10]. Another way to increase the affinity of immune interaction, successfully fulfilled in the number of recent developments, is the targeted design of antigen-binding parts of recombinant antibodies [41–43].

So, in a non-competitive analysis the use of high sensitive ways of registration of immune complexes or bound markers can potentially lead to significant lowering the detection limit (fig. 2). For example, the systems of cascade amplification of signal in immunoassay are developed actively, in which a marker bound with immune complex marker after separation of the reaction mixture components acts as the inducer of formation of a big number of molecules, which are detected at the final stage of the test. (The simple variant of such cascade is a traditional ELISA, which detects not molecule of enzyme bound with the antigen-antibody complex as it is, but the products of catalyzed reaction). Although theoretically such cascade or several cascades can significantly reduce the detection limit, the restrictor for

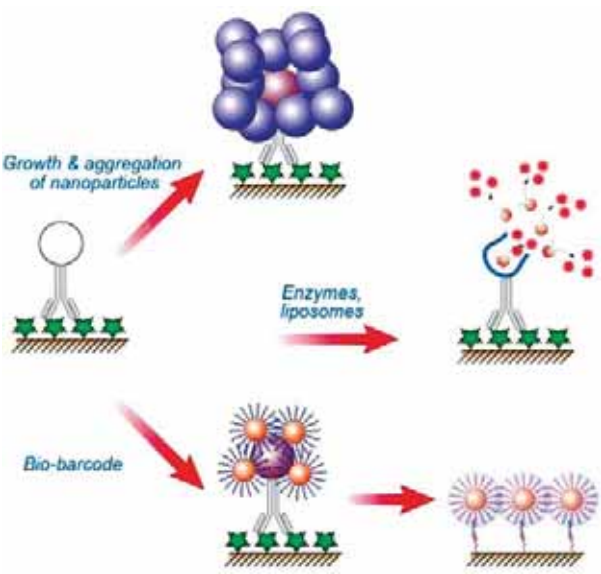

Fig. 2. **Appoaches for signal amplification in immunoassays**

this approach is non-specific binding, resulting in increase of background signal. Nevertheless, there is a number of developments in which strengthening of the signal assures detection of single molecules of antigen. For example, to reach this target can be used the formation of micro-dispersive colloidal complexes on the ground of a single molecule of a marker [44].

Highly sensitive assays may be realized also by PCR amplification of target nucleotide sequences [45–47]. The given nucleic acid lateral flow immunoassay allow to detect a few bacterial cells in grams of tested samples [45].

Evidently, that the limit of detection shall always be worse than the theoretically possible one, as not only registration of some signal is necessary, but also confirmation of difference of its level from non-specific interaction in the analytical system.

The ways of improvement of analytical characteristics of heterogeneous immunoassays

First of all, the sensitive heterogeneous immunoassay does not necessarily demand incubation of reagents till achievement of chemical equilibrium [48]. The simplest solution, which does not demand the changes of reagent bases and the means of detection is the reduction of duration of the assay stages. Traditionally recommended prolonged stages of ELISA (an hour and more for each immune stage) improve first of all reproducibility and precision of the assay. In the number of cases it is demonstrated, that incubation duration can be reduced to 10–15 minutes with not very big reduction of amplitude of detected signal and almost without alteration of the assay operating range [49, 50]. In accomplishment of kinetic assay its reproducibility starts to play important role, demanding, for example, strict match of the times of incubation for all samples of tested series. However, modern means of automation [51], used in the number of immune enzyme analyzers, allow fulfilling this task. Regarding heterogeneous immunoassays it should be considered that the reason for their extended duration is not heterogeneity of interaction (between reagents presenting in the solution and immobilized at the surface of carrier), but slow diffusion-controlled processes of establishing of equilibrium between the layers of fluid, locating at different distances from a carrier. Considering this, promising are the developments, in which this diffusion exchange is significantly accelerated and reagent medium is structured in the way that time losses due to diffusion become insignificant.

For mixing of pre-surface layers and the total volume of fluid ultrasound processing recommended itself well for the number of test-systems demonstrating 2–5-fold reduction of the analysis duration [52].

An effective solution of the problem of diffusion limitations is transfer of interactions in microvolumes, which is possible with the use of highly sensitive systems of detections of markers (for example, modern fluorescent detectors) [53–55]. Such miniaturization is used in many developed immunochips. Immunoassay at a chip on sequence of stages matches classic solid-phase immunoassay. The principle difference is that antibodies monolayer is immobilized at the surface of silicon, quartz or polymeric materials (Teflon, polycarbonate) [56] of about 100 μm^2 in area. To compare — the area of only a bottom of the well of a standard 96-well plate for ELISA comprises $3 \cdot 10^7$ μm^2, i.e. 300 thousand times greater ([36]. Currently immunochips are usually used for multiparametric tests, in the frameworks of which small areas of binding are incubated till achievement of equilibrium with the total sample volume. In this case achieved immunochemical reaction remains to be diffusion-controlled, and performance of the test requires several hours [57]. Incubation of reagents directly in the areas of binding by the formation of thin layers of fluid allows sufficient reduction of time for formation of detected complexes. As a result

the duration of the assay can comprise 10–15 minutes, corresponding modern demands to express analysis [58, 59].

Significant limitation for the introduction into practice of immunochip technology yet 5–10 years has been the involvement of complicated and expensive optical equipment — confocal microscope, CCD-camera, etc — for highly sensitive detection of a tagged compound in the quantities, which can bind at a small area of a chip. At the moment to solve these tasks relatively cheap portable devices have been invented, which shall assist more rapid introduction of immunochips into clinical practice [58, 60].

Reduction of duration of heterogeneous immunoassay is also reached by its transfer into the flow mode with the use of cells of small diameter. Flow-injection analysis, suggested by Ruzicka and Hansen [61], is grounded on automatic injection of fixed volume of a sample into continuous flow of a buffer solution. Carriers (sorbents) with immobilized antibodies are used to separate detected specific complexes in flow-injection immunoassay, and a detector registers product of enzymatic reaction after binding of enzyme-marker with the sorbent. The use of flow-injection systems for immunoassay incites the transfer of immunochemical and enzymatic reactions into kinetic mode, reducing the contact time to several minutes and respectively reducing the duration of the assay. The necessary conditions for obtaining of reproducible data in kinetic mode is stability of such parameters as the contact time of reagents, temperature and the volume of analyzed sample. In this respect for flow-injection immunoassay principal significance has the availability of serially produced devices for automatic performance of all injection manipulations.

Membrane immunochromatographic assay, the brief specification of which was presented in the section 2, can also be qualified as a variant of flow heterogeneous immunoassay. In a classical variant the main limitation of immunochromatography is low sensitivity due to the use of colloidal dyed particles as directly detected markers. In recent years a number of developments have been suggested, which overcome this limitation and accomplish amplification of detected signal (see section 3), or transfer to alternative markers, revealed in lower concentrations [32, 62]. Thus the number of works depicts the option of more sensitive detection in the membrane assay of colloidal semiconducting fluorescent markers [63, 64]. Significant reduction of detection limit can be achieved due to transfer from optical detection of coloured markers to the registration of their other physical properties, for example, electrical and magnetic. Thus, highly-sensitive immunochromatographic determination of troponin with the use of magnetic nanoparticles is described in [65]. Several immunochromatographic systems with electrochemical registration of markers are suggested [66–68], confirming the potential of this approach. The Table summarize several example of the application of different labels in rapid tests.

Priority labels for rapid immunoassays

Label	Example(s) of application
Spheric gold nanoparticles	[97, 98]
Other gold nanoparticles	[99, 100]
Liposomes	[101, 102]
Gold nanoparticles + marnetic particles	[103]
Different nanoparticles	[104–106]
Fluorescent dyes	[107, 108]
Marnatic particles	[109, 110]
Quantum dots	[64, 111, 112]
Lanthanides	[113, 114]
Latex particles	[115]
Upconverting phosphor	[116, 117]
Eu(III) chelate microparticles	[118, 119]

Homo+heterogeneous immunoanalytical systems

Considering benefits and disadvantages of homogenous and heterogeneous immunoanalytical methods it is considered advisable to combine in one scheme rapid formation of immune complexes in the solution and the efficacy of their detection in heterogeneous systems. A promising approach, assuring achievement of this target is implementation of polyelectrolytes in immunoassay. Polyelectrolyte separation in immunoassay can be performed on the ground of:

- Interaction of counterions (polycation-polyanion) pair [69, 70];
- Interaction of polyelectrolyte-ion of metal pair [71];
- Monomer polymerization [72–76].

The first variant of separation was performed with the use as counterions of linear water soluble polyelectrolytes: polyanion — polymethacrylic acid, polycation — poly-N-ethyl-4-vinylpyrydinia or poly-N-N′-diethyl dyallialammonium. These polymers interact

with each other in a wide range of conditions almost immediately forming an insoluble complex. Due to this, having obtained a conjugate of one of polyelectrolytes with an immunoreagent, we may perform detection of analyzed antigen in solution as in case of usual rapid homogenous methods, as then, with the addition of counterion into the system, to separate rapidly formed immune complexes (Fig. 3).

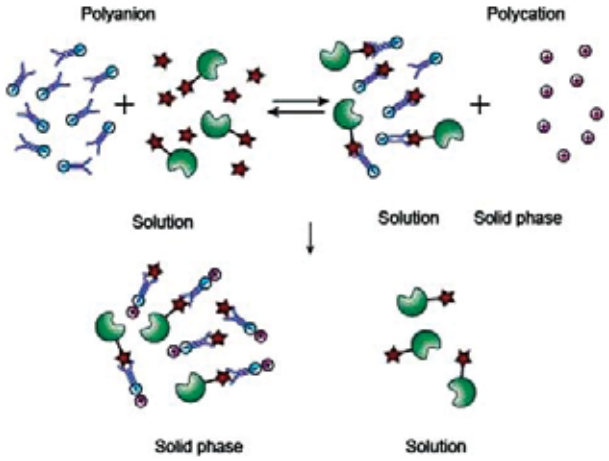

Fig. 3. **Principle of polyelectrolyte separation in immunoassay**

Performed comparison of traditional solid-phase ELISA and ELISA on the basis of polyelectrolytes for detection of such compounds as insulin, testosterone, immunoglobulins, hepatitis B surface antigen demonstrated that the use of polymeric carriers allows reducing the time of analysis from 2–3 hours to 15–30 minutes without losses in sensitivity and specificity [77].

The second variant of separation implements the polyelectrolyte-ion of metal pair. The procedure, suggested by Auditore-Hargreaves K., is based on the use of polymeric carriers, solubility of which depends from the presence of cations [72]. Water-soluble polymers can be precipitated, for example, with the reduction of pH or adding of such ions as Ca^{2+}, and repeatedly transferred into the solution — by the increase of pH and adding of chelating agents (ion-citrate, ethylendiaminetetraacetic acid, etc). Developed is homogeneous immunoassay on the basis of algic acid, which assures 30 minutes identification of antigens, molecular weight of which is within the range from 100 to 50000 Da [72].

The third variant, suggested by Hoffman et al, is grounded on the generation of a solid polymeric phase from soluble monomer. On the basis of this principle systems of immunoglobulins detection with free-radical and temperature initiation of polymerization are developed. The first system (polymerization *de novo*) is grounded on the use of two types of antibodies conjugates: antibodies(1)-fluorophor and antibodies(2)-organic monomer [73–75, 78]. After completion of immunochemical reaction the reaction of polymerization is initiated by free radicals. In the results of polymerization insoluble polymeric particles are formed, content of the marker in which is proportionate to the quantity of antigen in the sample. The second system is based on implementation of temperature-dependent polymers [73, 79]. Here, the same as by polymerization *de novo*, two types of conjugated antibodies are used. Formation of specific immune complexes happens at the temperature lower the critical one for the given polymer, and increase of temperature allows separating the complex from the solution, by this the number of marked antibodies, included into precipitate, is proportionate to the content of antigen in the sample. The most promising for this immunoassay are polymers and co-polymers of acrylamide with N-alkyalacrylamide.

Poly-N-isopropylacrylamide and its co-polymers, except for thermal precipitation, can be precipitated by 14–20% ammonium sulfate. Free IgG at do not precipitate such concentrations of salt. So this method allows separating free immunoglobulins from conjugated with polymer ones.

On the ground of this principle there immunoanalytical methods with the use of enzymatic and fluorescent markers were developed. They demonstrated efficiency for the detection of immunoglobulin G, hepatitis B surface antigen, *Chlamidia trachromatis* etc. [73, 80].

Together with interpolyelectrolyte interaction, an efficient solution, combining all benefits of homogeneous and heterogeneous immunoassays, is the implementation of magnetic immune sorbents [81–83]. The use of these particles as a solid phase in immunoassay gives the possibility to increase significantly the area of surface to immobilize reagents, distribute them equally along the total volume of the reaction medium, hence accelerating heterogeneous interactions. By this after completion of the first stage of the assay implementation of outside magnetic field assures simple and rapid separation of reagents. The use of magnetic colloidal particles (MNP) as a solid phase in ELISA allows improving its analytical characteristics. There is a number of works on MNP implementation

in ELISA for the detection of compounds of various nature — pesticides, hormones, mycotoxins, allergens, proteins, viruses, bacteria [84–87]. By this the duration of specific interactions can be reduced to 5–10 minutes, and the analysis in general up to 20–30 minutes. It is important also that magnetic separation allows performing pre-concentration of target compound from a big sample volume. This concentration allows additional reduction of the detection limit of the target compound 1–2 orders [87].

Ordinary centrifuging can be also used to separate immunosorbent from the reaction medium. This approach is accomplished in the number of test-systems with the use of antibodies, immobilized at the particles of high-disperse latex, usually — polystyrene (which allows using standard protocols of absorption immobilization being developed for ELISA) [88].

Homogeneous immunoanalytical systems

Implementation of homogeneous methods of immunoassays is significantly limited with the influence of sample components on registered signal, due to which it is complicated to distinguish this influence from specific complex formation. Pretty solution of this problem is the use of differential measurements in the assay, when results of measuring with specific immune reagents and with antibodies with the compound, knowingly not present in the test sample, are compared [89].

Nevertheless, solutions with minimal influence of matrix on the result of the assay are preferable — despite the absence of the separation of reaction mixture components before measurements at homogeneous assay. In this respect significant interest is drawn by new markers and new ways of immune complexes registration. Thus, the application of lanthanides complexes as markers allow to reduce impact of background signal due to possibility of prolonged registration and integration of fluorescence [90, 91]. An alternate approach is the use of long wavelength (600–1000 nm) fluorescence that also reduces background impact from sample constituents [92–94].

One more promising approach is the use of the signal, generated by close spatial approach of interacting immune reagents. For example, a number of immunoassay systems was described, grounded on the effect of fluorescence resonance energy transfer (FRET) [95, 96], occurring by the approach of two markers, conjugated with antibody and antigen (Fig. 4).

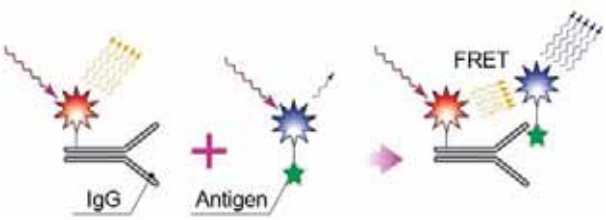

Fig. 4. **Principle of FRET-based immunoassay**

Conclusion and Prospects

As the presented brief analysis shows, the developments, targeted at increase of analytical characteristics of immunochemical test-systems are extremely diverse. The efforts of the researchers are not concentrated on acceleration of sensitivity or increase of sensitivity of rapid tests, but suggest various solutions within the field of options, depicted at the fig.1. In this respect it is complicated to make exact forecast regarding more promising directions of immunoassay. However, it should be expected that the principle of multilevel diagnostics, including preliminary screening and further confirming test shall preserve in near future. Due it can be also expected the preservation of two clusters — more rapid and more sensitive analytical methods. However, the introduction into practice of developments, related with analytical markers, new methods of assay performance and registration of immune complexes shall lead to substitution of conventional methods with new ones. Modern means of registration of ultra-small signals and data processing allows effective control over the content of compounds in samples in extreme low concentrations. By this, diagnostic decisions shall be made considering information about content of a big number of diagnostically significant compounds, which shall results into development of multiparametric analytical systems, as well as information about peculiarities of patient's metabolism, considered in diagnostic data bases within the frameworks of the shift to personified medicine. Simplification and increase of sensitivity of test-systems for field assay shall results into their active implementation in non-medical spheres.

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СУЧАСНІ МЕТОДИ ІМУНОХІМІЧНОГО АНАЛІЗУ: ПОЄДНАННЯ ЧУТЛИВОСТІ ТА ШВИДКОСТІ

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Огляд присвячено історії та перспективам розвитку методів імунохімічного аналізу. Докладно висвітлено переваги і перспективи використання антитіл як детектуючого агента, сучасні вимоги до методів імуноаналізу та передумови для формування двох груп методів (гомогенних експрес-методів з відносно невисокою чутливістю і гетерогенних високочутливих з більшою тривалістю поставлення), а також можливості поліпшення аналітичних характеристик цих методів. Обговорено прогнози найбільш перспективних напрямів подальшого розвитку методів імунохімічного аналізу, зокрема мультипараметричних аналітичних систем. Розглянуто можливість створення універсальних підходів імуноаналізу, що поєднують високу чутливість гетерогенних і швидкість поставлення гомогенних методів (наприклад, на основі поліелектролітів або магнітних колоїдних частинок).

Ключові слова: імунохімічний аналіз, гомогенні та гетерогенні методи.

СОВРЕМЕННЫЕ МЕТОДЫ ИММУНОХИМИЧЕСКОГО АНАЛИЗА: СОЧЕТАНИЕ ЧУВСТВИТЕЛЬНОСТИ И СКОРОСТИ

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Обзор посвящен истории и перспективам развития методов иммунохимического анализа. Детально рассмотрены преимущества и перспективы использования антител в качестве детектирующего агента, современные требования к методам иммуноанализа и предпосылки для формирования двух групп методов (гомогенных экспресс-методов с относительно невысокой чувствительностью и гетерогенных высокочувствительных с большей длительностью постановки), а также возможности улучшения аналитических характеристик этих методов. Обсуждены прогнозы наиболее перспективных направлений дальнейшего развития методов иммунохимического анализа, в частности мультипараметрических аналитических систем. Рассматривается возможность создания универсальных подходов иммуноанализа, сочетающих высокую чувствительность гетерогенных и быстроту постановки гомогенных методов (например, на основе полиэлектролитов или магнитных коллоидных частиц).

Ключевые слова: иммунохимический анализ, гомогенные и гетерогенные методы.

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INDIVIDUALIZATION OF CANCER TREATMENT: CONTRIBUTION OF OMICS TECHNOLOGIES TO CANCER DIAGNOSTIC

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Cancer is a disease, which explicitly illustrates success, failures and challenges of the modern biomedical research. Technology development has been the driving force of improvements in the cancer treatment. Introduction into clinical practice of genomics, RNA profiling and proteomics technologies have provided a basis for development of novel diagnostic, drugs and treatments. In this chapter, contributions of OMICs technologies to personalization of cancer diagnostic and treatment are discussed. The focus is on technologies that showed capacity to deliver diagnostic that may be used in the clinic as routine tests. Three clinical cases are presented to illustrate already available individualized cancer diagnostic.

Key words: personalized cancer medicine, genomics, transcriptomics, proteomics, metabolomics, diagnostic.

Why OMICS technologies are needed for treatment of cancer?

Cancer still kills people. It was easier to send a man in the outer space or to the Moon, than to improve survival of patients having advanced and metastatic cancers. This highlights complexity of cancer as a disease, which is apparently much higher than to build and launch a space rocket. Carcinogenic transformation of cells is accepted as the main cause of cancer [1–4]. Carcinogenic transformation is defined as a number of changes in the cell physiology, which lead to expansion of malignant cells in the body, corrupting the normal physiology, and ultimately killing the person.

The key conclusion of more than 50 years of intense studies is that the collected knowledge has not reached the critical mass required to find cure against cancer. The oncogenes and tumor suppressor model has been a great step forward [1–4], but today is clear that carcinogenic transformation of cells is the result of interaction of hundreds molecules. Out of the hundreds of these cancer-promoting genes, RNAs, proteins and metabolites many are the same as they are in the normal cells. It is their corrupted activity, mis-localization, and misplaced interactions that make them tumor-promoting (Fig. 1). This confusion has only underlined complexity of cancer.

A solution to the complexity problem has been proposed by introduction of technologies for comprehensive study of carcinogenesis. These technologies focused on studies of genomic DNA (genomics), RNAs (transcrip-

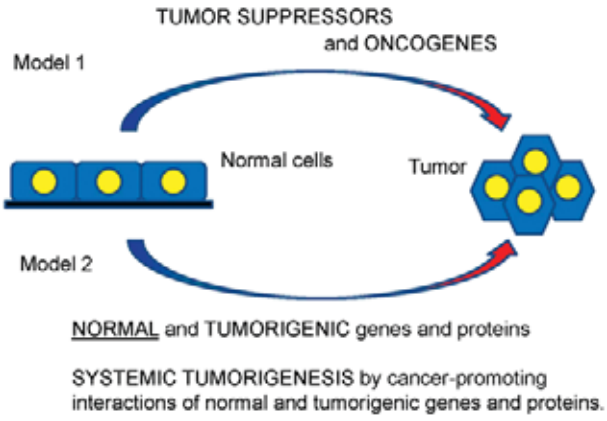

Fig. 1. **Two models of tumorigenesis**
The first model explains tumorigenesis by involvement of tumor suppressors and oncogenes (upper part). The second model explains tumorigenesis as a systemic effect of normal and tumorigenic genes and proteins (lower part). The difference is that the systemic tumorigenesis model postulates that even normal genes and proteins may contribute to tumorigenesis, if their interaction networks shift from the normal physiological reactions to the disease promotion.

tomics), proteins (proteomics) and metabolites (metabolomics) [5–7]. Historically, introduction of nucleotide microarrays to study expression of RNA was the first strong contribution to the comprehensive exploration of carcinogenesis [8]. Development of the microarrays was possible due to successes of technologies for synthesis of oligonucleotides and production of cDNA on a large scale and in automated way. A chip-printing technology was another component of the success. Development of sequencing technologies, especially of massive parallel sequencing, has given boost to comprehensive studies of genome for the clinical diagnostic [9]. Comprehensive studies of the proteome are still waiting for a wide use of intact protein analysis technology. Current technologies of mass spectrometry, 2D gel and other electrophoresis, or liquid chromatography are not providing quality that is required for full description of the human proteome [10, 11]. However, the situation may change with introduction of ZP-technology [12]. The least developed of the OMICs technologies is metabolomics. The high variability of physico-chemical and structural properties of the metabolites makes it challenging to detect and identify all metabolites by a single technology. Despite all shortcomings, OMICs studies have become essential for success in treatment of cancer, due to their ability to a comprehensive analysis. Therefore, there is no alternative to development of fast, reliable, informative and cost-efficient OMICs technologies for diagnostic and treatment of cancer.

Genomics

Since the discoveries that the genomic DNA carries hereditary information, and is the white-print of the most of the living creatures, study of genes, or genomics, has been a subject of intense developments. This excitement was translated in a slogan that «cancer is the disease of genes». The slogan's correctness is questionable today, as the non-genomic mechanisms may have a strong impact on tumorigenesis. How many genes do humans have? What is the structure of these genes, as introns and exons? What type and how many mutations are in the genome of a given patient? What are epigenetic changes in the genes? All these questions have importance for understanding of carcinogenesis, and subsequently for treatment of cancer.

In this section are discussed technologies for studies of genome, which may have a value for clinical applications (Fig. 2). These technologies have been developed to the extent that they may be applied in the clinic for diagnostic, selection of treatment and monitoring of response of a patient.

Massive parallel sequencing (MPS/NGS)

The excellent research on biochemistry of DNA paved the way to development of DNA sequencing techniques. The first generation

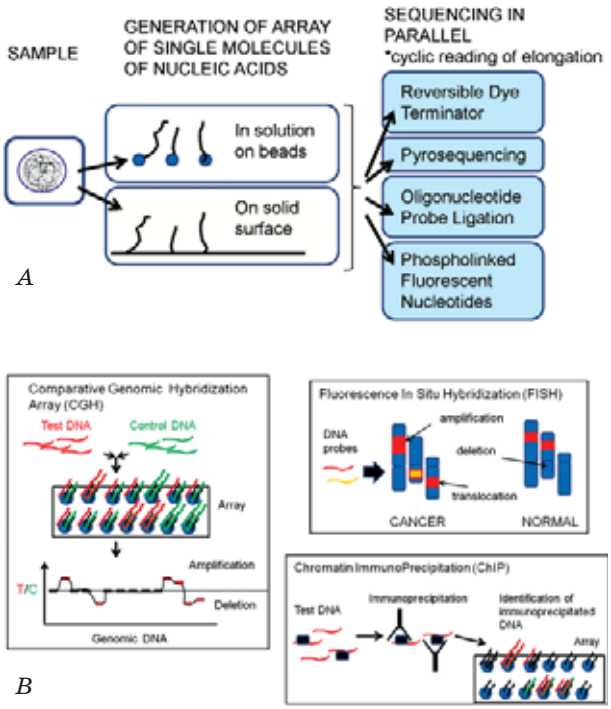

Fig. 2. **Presentation of genomics and transcriptomics technologies that may be used in clinical diagnostic:**

A — Massive parallel sequencing technologies. Two main parts of MPS are indicated. The first is preparation of nucleic acids for sequencing reactions. This is done by preparing single molecule suspensions, or by anchoring nucleic acids to a solid surface. The second part is enzymatic reactions to read the sequence. The reactions include polymerase or ligase reactions, and the sequence reading is by detecting incorporation of defined nucleotides which are added to the reaction in cycles.

B — Schematic presentation of CGH array, FISH and ChIP technologies. Amplifications or deletions of genomic DNA are detected by CGH arrays as increased or decreased relative signal of the test DNA in comparison to the control normal DNA. Binding of specific DNA probes to the chromosomal regions in FISH assays indicate regions of amplifications, deletions and translocations. For ChIP, immunoprecipitation of specific regions of DNA allows detection and profiling of only these regions, for example detection of transcriptionally active regions, or DNA regions interacting with specific proteins, or epigenetically silenced regions.

DNA sequencing methods are Maxam-Gilbert fragmentation and Sanger's dideoxy base-termination techniques [13, 14]. However, these techniques in their original forms were too cumbersome for being used in the clinical practice. The step toward clinic was by introduction of automated sequencers [15]. Automation allowed to detect gene mutations of the clinical importance. However, it was still far from a comprehensive analysis of the whole genome of a patient in the routine clinical practice. Faster and affordable methods were needed, and they come with development of massive parallel sequencing (MPS), known also as the next generation sequencing (NGS).

MPS is based on parallel sequencing of short fragments of DNA, which are then aligned to produce gene sequences (Fig. 2, *A*). The size of sequenced fragments is from 30 to 700 bases, depending on the sequencing method and instrumentation [16, 17]. This relatively short length of the sequenced fragments imposes limitations on the quality of definition of the complete genes sequence.

MPS has been used successfully for analysis of mutations in genes, with the emphasis on the exon analysis. Focus on exons allows generation of data which could be used in clinical diagnostics within relatively short assay time. As an example, the full exon sequencing and detection of the mutation profile of a tumor cells may be completed within 30 days [18, 19]. The second example is the contribution of MPS to profiling of mutations in different sub-types of cancer, providing insights into molecular heterogeneity of tumors [19]. Understanding this heterogeneity is essential for development of personalized treatment of patients. The expectation is that MPS will become a standard and routine examination of cancer patients. The nearest years will show whether this expectation will indeed be realized in better treatment of patients.

CGH, PCR, FISH and ChIP tests

Pre-MPS era had given rise to a number of methods to assess structure and mutations of the genes (Fig. 2, *B*). Comparative Genomic Hybridization Array (CGH) was used to detect gene aberrations on the whole genome level [20]. However, the resolution power of CGH arrays has been in the range of 5 kB to 0.2 kB, and variations in the gene structure have been the core information delivered by CGH [20].

Fluorescence in situ hybridization (FISH) is used to detect rearrangements of selected genes, e.g. deletions, amplifications and translocations [21, 22]. Clinical application of FISH is limited by its low number of monitored DNA fragments, and relatively large work-load for performing the test. Multiplexing FISH by using different probes with different detection wavelength, and use of nano-devices to minimize and automate the test are 2 developments which make FISH still useful in the clinical diagnostic [21, 22].

Polymerase-chain reaction (PCR)-based analysis of the genomic DNA is used less and less in the clinical diagnostic. The niche for PCR has become analysis of pre-selected mutations [23]. However, PCR is more used for analysis of RNAs than genomic DNA. Chromatin Immuno-Precipitation (ChIP) has proven the high informative value in studies of chromatin re-arrangements and methylation of the genomic DNA [24]. Therefore, the unique information which may be delivered by ChIP tests is the profile of epigenetic changes in the genome. On the other hand, complexity of the ChIP tests limits its clinical applications.

Complexity and low automation level of the CGH array, FISH, PCR, and ChIP tests are major hinders for their use in routine clinical diagnostic (Fig. 2, *B*). Cost efficiency of these assays is also lower, as compared to tests with the recent developments of MPS. Therefore, each of the genome profiling technologies will have their niches. MPS will with high probability dominate the whole genome profiling, while CGH, FISH, PCR and ChIP tests will focus on selected genes and genome areas.

Transcriptomics

Historically, mRNA profiling by expression arrays has been the first true OMICs technology. The ground of this technology was laid by excellent works on the biochemistry of oligonucleotides and generation of cDNA. PCR-based analysis of mRNA expression was competing with the RNA expression arrays, but PCR was inferior due to the higher complexity and lower robustness. mRNA profiling has also been proposed for the clinical diagnostic. However, arrival of the massive parallel sequencing technologies has given the real boost to RNA profiling by providing flexibility, speed and additional information about mRNA, microRNAs and long non-coding RNA, e.g. expression and mutations [25].

Massive Parallel Sequencing of RNAs

MPS technologies used for profiling of RNAs are similar to those used for profiling genomic DNA, but the focus is on mRNA,

siRNA/miRs, and lncRNA. The difference is only in preparation of samples for analysis [26–29]. RNAs are more sensitive to degradation, located in nuclei and cytoplasm, and have different sizes, as compared to the genomic DNA. These features make challenging MPS of RNAs, as variability in quality of the samples would be reflected in discrepancies of produced data. Despite the challenges, information delivered by MPS of RNAs allows better insight into molecular activities in the tumors. A number of examples confirmed value of RNA MPS for making clinical decisions [30, 31]. For example, MPS sequencing of RNAs in tamoxifen-resistant breast cancer cells identified 1728 RNAs associated with the resistance. This number of the affected RNAs indicates that the acquisition of the tamoxifen resistance is a complex process, with involvement of many activities. On the other side, this study opens for better monitoring of the resistance, and the most important, it provides the basis for selection of more efficient treatment by combined blocking of the key RNA-related regulators of the resistance [31].

Expression arrays

RNA expression arrays are undergoing evaluation of their use in the clinical diagnostic. Only 5 years ago, RNA expression arrays were at the leading edge of entering clinical diagnostic. The limitation at that time was not in the technology itself, but in applicability of the generated information for diagnostic and making decision about treatment. While measuring expression of RNA provided large volumes of information for research purpose, this information was difficult to translate into diagnostic and prognostic values. The reasons were discrepancies between mRNA expression and expression and activity of the corresponding proteins. Another critical limitation was not sufficient robustness of the arrays. As an example, RNA microarrays from different suppliers could produce different detection values for the same RNAs [32].

The niche for RNA expression arrays is changing from the all-gene coverage approach to measuring a set of RNAs of importance for specific type of cancer or a set of cancer drugs. Such arrays are combined now with dedicated systems biology tools to extract disease-relevant information. For example, the arrays have been used to identify long non-coding RNAs associated with breast cancer [33]. MicroRNA arrays are another novel niche-approach that may be the way to discover cancer-associated microRNAs [34].

PCR-based analysis

PCR-based RNA analysis is in the situation similar to RNA expression arrays. Notably, PCR-bases analysis is not anymore considered for a comprehensive full-transcriptome screening of RNA expression. PCR-based analysis is currently used for measuring defined sets of up to 100 different RNAs, predominantly mRNAs. For example, focused analysis of expression of the key genes involved in acute myeloid leukemia unveiled 19 up-regulated and 25 down-regulated genes [35]. An important advantage of the PCR-based assays is their technical simplicity. Such assays may be used even in a small size laboratory, and for the low cost. Introduction of companion diagnostic into clinical practice also contributes to the niche-development of PCR-based tests. As examples, PCR-based tests of mutations in BRAF, EGFR, BCR-ABL, PDGFRs and MEK1 genes are proposed to the clinic as companion diagnostic of drugs acting on these kinases [36, 37].

Thus, MPS technologies have begun to dominate a comprehensive RNAs profiling, while RNA expression arrays and PCR-bases assays are specializing in measurements of pre-selected sets of RNAs. It has to be notes that the recent developments of systemic analysis tools have strongly contributed to extraction of information useful for clinical diagnostic, prognostic and selection of treatment.

Proteomics

All known anti-cancer drugs act directly on proteins or require proteins for their activity [38]. This makes proteomics essential for diagnostic and selection of treatment. Early approaches of monitoring expression of the drug targets have shown their positive contribution to cancer diagnostic and treatment. Such approaches are currently a standard of routine diagnostic, and are applied overwhelmingly by using immunohistochemistry (IHC). Measurements of the expression of Her2, EGFR, ER, PgR in tumors by IHC are standards when an oncologist has to decide about use of Herceptin, Iressa, or antihormonal therapy [39]. The drawback of such approaches is in the limited number of measurements, as a single assay measures only one protein. This is why a comprehensive profiling of the whole proteome has been expected to improve diagnostic. As the minimum, the clinical oncologists need to evaluate a drug target itself, and all proteins which may affect drug

efficacy [40]. This requires to measure in a single assay up to thousands of proteins. This is why proteomics experiences strong pressure to deliver good diagnostic tools.

Proteomics technologies may be classified on the basis of what they study, e.g. proteins, peptides or epitopes, how they separate the analytes, and what they detect to identify proteins. Studying proteins or their products peptides, or protein epitopes, are the main cut-off points for deliverables of the proteomics technologies (Fig. 3). Peptidomics approaches by the Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC-MS/MS) have recently been popular [41, 42]. However, LC-MS/MS is not capable to detect and identify the intact proteins and their isoforms. Therefore, there were too low correspondence of claimed identification of proteins, and the real impact of these results on the clinical diagnostic [42]. 2D gel electrophoresis (2D-GE) has been the technology to study intact proteins [43–45]. However, 2D-GE has limited protein separation capacity. Novel intact-protein proteomics techniques have been under development [12, 43]. The protein arrays are considered of being closest to enter routine clinical diagnostic. Proteomics has also other technologies for separation and identification of proteins, which are too laborious to be used in clinic. Therefore, in this section I focus on proteomics technologies which may have an impact on the clinical diagnostic, intact-protein proteomics, peptidomics and protein arrays (Fig. 3).

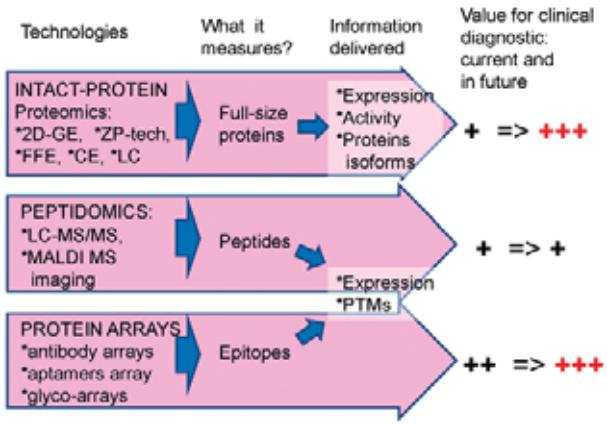

Fig. 3. **Proteomics technologies that may be used in clinical diagnostic**

Proteomics technologies to study intact proteins, peptides and protein epitopes are indicated. What these technologies measure, and type of delivered information are mentioned. Pluses indicate current and expected in the nearest years levels of application of the technologies in the routine clinical diagnostic.

Intact-protein proteomics

The key feature of the intact-protein proteomics is that the studied analytes are proteins. Therefore intact-protein proteomics is the most informative profiling of the proteome among all proteomics technologies. Despite such importance, technologies of the intact-protein proteomics have not had capacities to describe the whole proteome. Variability of physico-chemical properties of proteins has been the main barrier.

2D-GE has been the most common technique of the intact-protein proteomics. Classical 2D gels may separate up to 5,000 proteins in one gel. pH zoom-in gels were reported to separate between 7,000 and 10,000 proteins [43–46]. Recently developed ZP-technology may be the breakthrough of the intact-protein proteomics, with its capacity to separate more than 20,000 proteins in a single run [12]. Separated proteins have to be identified, and today the best identification technique is mass spectrometry. MALDI TOF mass spectrometry has been successfully used in combination with 2D gels.

2D-GE and ZP-technology have been used for cancer diagnostic in the clinic (see examples in the section #6 below). However, these techniques are rather labor intensive, and are not enough well automated for routine use in a clinical diagnostic laboratory. The use of these techniques has been in the translational cancer research, and in managing a limited number of patients. To be suitable for use in the routine clinical diagnostic at a large scale, an instrument with fully automated manipulations would be required. For the moment, all attempts to create such an instrument have not been successful.

Other techniques employed in the intact-protein proteomics are free-flow electrophoresis, capillary electrophoresis and liquid chromatography [46–49]. However, none of these techniques is capable to detect all proteins of the human proteome, and provide sufficient separation of even those proteins that were detected. Significant efforts have been invested in development of protein mass spectrometry. However, mass spectrometry is unlikely to succeed in analysis of full-length proteins due to limitations of the ion optics and physical properties of sub-atomic particles. Therefore, significant developments have to be made to prove that these techniques have a future in the clinical diagnostics.

The intact-protein proteomics has another advantage over other proteomics technologies, as it allows unbiased analysis of protein activities.

For example, it allows detection of all kinases which may be inhibited by a tested kinase-inhibiting drug [50]. The comprehensiveness of the tests means that it would be analyzed not only intended target of the drug, but also all kinases in the tested tumor. This provides information about efficacy of the drug, and about potential off-target and side-effects. Among other activities which can be tested by the intact-protein proteomics, there are acetylation, ubiquitylation, PARylation, proteolysis, and glycosylation [51]. Taking into account introduction in the clinical practice drugs affecting these post-translational modifications, the diagnostic value of the intact-protein proteomics is going to increase.

Peptide-based proteomics

Peptide-based proteomics is strongly associated with use of mass spectrometry [41, 42]. The focus of mass spectrometry on peptides is because of 2 limitations. The first limitation is due to biochemistry of proteins, and the second is due to the physics of ions and sub-atomic particles. The biological limitation is because of the enormous complexity of the mass spectrum of an intact protein. Many combinations of isotopes distribution in an intact protein, and multiple charge ionizations of the protein make it challenging to obtain a well defined protein spectrum of the high resolution. On top of that, if there is a mixture of proteins with the molecular masses in the same range, separation of these proteins by mass spectrometry would be very difficult to achieve. The physical limitation is imposed by changing mass values of proteins in different conformations and as polymers. Atomic interactions change the total energy of the protein as compared to the sum of masses of the amino acids. The energy change is relatively low for peptides, and does not have a significant effect on the mass value of the peptide. However, for the molecules of mass higher than 20,000 daltons, the change of energy may be in the range of 900 MeV. This may be translated in the mass change corresponding to more than 0.7 dalton, as 1 atomic mass unit is equal to 931,49 MeV. Such uncertainty of the mass of large molecules makes irrelevant applications of the high resolution mass spectrometry. What is the reason to rely on the resolution of less than 0.001 dalton, if the uncertainty of masses due to the large size of the intact proteins is of more than 0.7 dalton?

However, mass spectrometry has a potential to be used in the clinical diagnostic. The niche of mass spectrometry-based diagnostic is in measurements of a limited set of peptides, which then would serve as biomarkers. The types of peptides to be measured define type of mass spectrometry technology. For example, MALDI TOF mass spectrometry is used to detect peptides ionized from a tumor section [52]. While detection of the peptides flying in the instrument is rather robust, the challenge is to control ionization of the peptides. Variations in ionization are due to differences in composition of the tissue in its different areas, thickness of the section, and slightest differences in application of the matrix. Variable ionization efficiency generates false differences in distribution of the measured peptides in the tissue. Proposed solutions with using internal controls may help to overcome the ionization problem, but it will complicate the technology. Such a complication would have a negative impact on the clinical applications of MALDI MS imaging. Other issues of MALDI MS imaging are technical problems of the size of the laser beam, speed of the spectra acquisition, and processing of the collected data [52]. All together, it makes that MALDI MS imaging is not yet ready to enter routine clinical diagnostic.

However, when peptides are the biomarkers, mass spectrometry may be the method of choice. As an example, detection of peptides in brain may have a diagnostic value [53]. With controlled sample preparation methods and internal MS standards, mass spectrometers may become standard equipment in the clinical laboratory. In 2013, the cost of a good quality mass spectrometer is affordable for specialized diagnostic laboratories. Another important initiative is generation of the Peptide Atlas, which provides peptides that may be used as internal standards [54]. When such peptides added to the samples, it provides a reference of the quality of analyte detection, and for the quantification of the marker peptide in the sample. Thus, peptide-based proteomics is dominated by mass spectrometry, and may have use in diagnostic based on detection of peptides as markers.

Protein arrays

Among all proteomics technologies, protein arrays are the most advanced on the way into routine clinical use. Protein arrays measuring expression of a defined set of proteins are the most probable candidates for the routine diagnostic [55–57]. The protein array technology is well developed, and it has been extensively tested. To generate a protein array, it is required to select name of proteins

and epitopes to be detected. Generation of capturing agents, e.g. primary antibodies, detection reactions, e.g. secondary antibody and a signal generation system, and selection of a format for the array — all these steps can be done with the already available and robust methods.

Thus, the strong side of protein arrays is the well established technologies and knowledge of the targets to be measured. The weak side is the need to ensure stability and specificity of the capturing molecules, e.g. antibodies. Another weak side of the protein arrays is the lack of unbiased analysis. With the arrays, one gets an answer only to questions about pre-defined proteins. If there is an off-target effect, it would be difficult to identify it with the arrays. Therefore, protein arrays may be a great tool for companion diagnostic, when measurements of up to 100 targets are required for diagnostic.

Metabolomics: mass spectrometry and nuclear magnetic resonance spectrometry

Metabolome represents very diverse set of chemical compounds in the human body. Nucleic acids and proteins have single polymeric structures of nucleotides or amino acids, respectively, even if the monomers may be quite diverse. However, metabolites are much more diverse as chemical structures. Metabolites are different types of polymers, e.g. glycans and lipids, and non-polymeric low molecular weight organic and inorganic molecules [58, 59]. This chemical diversity of metabolites creates a big challenge for their comprehensive analysis.

On the other side, metabolomics holds promise of being of the high importance for diagnostics. Robust metabolome profiling of patients would contribute to prediction of drug pharmacokinetics, and response of the body to treatments. Metabolic syndrome and metabolic insufficiency are among the most serious complications in treatment of cancer. Metabolome profiling would provide a tool to predict response to treatments, and therefore has its role in the clinical diagnostic.

Nuclear magnetic resonance (NMR) and mass spectrometry are two techniques which dominate studies of the metabolites [58–60]. However, none of these techniques have yet capacity to be incorporated in the routine clinical practice. NMR has the best efficiency in identification of metabolites, but sensitivity has not been sufficient for application of NMR to small quantities of clinical samples. Notably, the quantities of metabolites in clinical samples are estimated at the picomolar level, while NMR requires micromolar quantities [58–69]. Mass spectrometry has higher sensitivity, but the drawback is in identification of ionized metabolites. Ionization degree and stability, or rather instability, of metabolites upon ionization limits coverage and identification of metabolites, and therefore application of mass spectrometry [58, 60]. The physical principles of NMR and MS, and the physico-chemical features of metabolites suggest that NMR may take the lead. This is because physics of NMR allows enhancing sensitivity, and it may pave the way to the breakthrough in metabolome-based diagnostic.

Integration of OMICs technologies in personalized cancer diagnostic: examples of applications

Clinical OMICs technologies are at different stages of development, and therefore deliver results of different quality. For diagnostics, the quality is defined by the type of measured analytes, comprehensiveness of the coverage of all analytes, sensitivity of detection, suitability of primary data for an analysis, and ability to deliver results to support diagnostic and selection of treatment. In this section, I would like to give examples of how OMICs technologies may help in management of patients.

When a patient arrives to a Hospital, decision has to be made about type of diagnostics to be applied. The samples for diagnostics may be blood and/or tumor biopsy as a surgically resected tumor or as a needle biopsy. The blood is used for preparation of circulating tumor cells (CTC), immunological tests, and use of the blood cells for mutation analysis of selected genes. The tumor material is used for preparation of an organ culture, primary tumor cells, and for biochemical tests.

Functional Molecular Diagnostics (FMDx) evaluates responsiveness of individual patient's tumors to different drugs by testing responsiveness of the living tumor samples in organ culture (Organ Culture FMDx), testing targets and modulators of the drugs' action (Functional Biochemical Assays), and by unbiased testing of the tumor's proteome profile (Proteomics FMDx). These assays measure in a real time how the patients' tumor may respond to different drugs before the patient is offered treatment, and whether the tumor is of an aggressive type (Fig. 4).

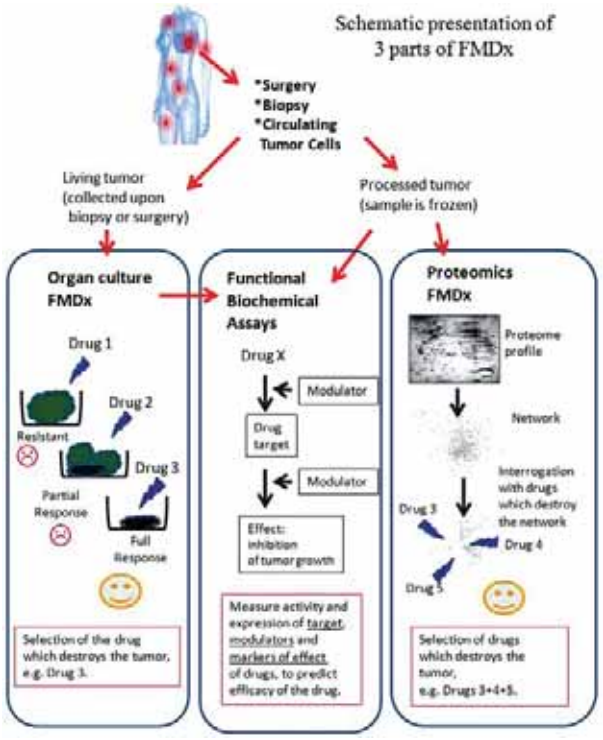

Fig. 4. **Overview of Functional Molecular Diagnostic**

The 3 components of FMDx are shown. The components are Organ Culture FMDx, Functional Biochemical Assays and Proteomics FMDx. Organ Culture FMDx is performed with living tumors, and evaluates sensitivity to drugs. Functional Biochemical Assays are used to evaluate mechanisms and efficacy of the drugs. Proteomics FMDx allow unbiased analysis of the molecular profile of the tumor, including diagnostic, prediction of tumor aggressiveness, and selection of drugs acting on the tumor.

In this section, examples of diagnostic with use of OMICs technologies are discribed. The Functional Molecular Diagnostic (FMDx) was developed to help oncologists in diagnostic and selection of treatment. FMDx consists of a number of tests to evaluate a molecular profile of a tumor, predict development of the disease, and select treatment tailored to the patient. The presented here examples describe 3 clinical cases. For the reason of the patients' integrity, no personal details are provided. The descriptions are to illustrate how OMICs technologies were integrated in the diagnostic efforts.

Case 1. Personalization of treatment with Proteomics FMDx

A lump was detected in both breasts of a woman. The lumps were removed surgically. No spreading to the lymph nodes, and no metastases were detected. The question of the oncologists was whether these 2 tumors were related, i.e. primary tumor in one breast and metastasis in the other, what is prediction of aggressive development of the disease, and which drugs would be most suitable for this woman.

The aliquots of the lumps were prepared for the proteomics study, by extracting proteins and performing proteome profiling (Fig. 5). 2D gel electrophoresis and mass spectrometry were used for generation of the proteome profiles of the tumors. The intact-protein proteomics was applied, which allowed detection of multiple isoforms of the tumor proteins. MALDI mass spectrometry was used to identify proteins, and the systems biology was used to build a network of relations between the tumor-related proteins. The network topology analysis and exploration of functional domains represented by the network, indicated that the tumors were of the similar profile. It means that the tumors in both breasts had the same origin, suggesting metastasis. However, the proteome profiles showed that the tumors were not of the aggressive type, and therefore unlikely that the disease would relapse. Immunohistochemistry tests did not provide conclusive basis for selection of chemotherapy or adjuvant therapies. Therefore, the results of Proteomics FMDx ensured oncologists that the most efficient treatment would be by applying tamoxifen, and restrain from chemo- and other adjuvant therapies. The woman has been regularly monitored for recurrence. Thus, for this patient, combination of the proteomics and systems biology, allowed to conclude about prognosis of the disease development, select the most suitable treatment, and preserve quality of life.

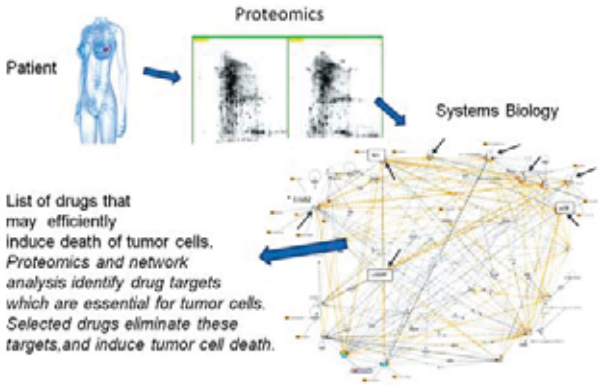

Fig. 5. **Workflow of Proteomics FMDx**

The tumor sample from the patient is subjected to proteome profiling, which then is analyzed by the systems biology tools. Systemic analysis identifies regulatory mechanisms deregulated in the tumor, and identify drugs which would have a tumor-eliminating effect.

Case 2. Personalization of treatment with Organ Culture FMDx

A patient was diagnosed with metastatic pancreatic cancer. Surgery was not applicable, due to multiple metastases. The oncologist required information about efficiency of drugs which were considered for treating this patient.

Biopsies of the primary tumor and metastases were collected. Organ culture samples were prepared immediately after resection of the tumors. Organ culture samples were prepared by using FMDx proprietary technique, and were exposed to drugs. The oncologist was interested in response to 5-fluorouracyl, oxaliplatin and gemcitabine. Two additional drugs were also tested. The first is an inhibitor of EGF receptor kinase, Iressa, and the second drug is an inhibitor of TGFβ type I receptor kinase, SB431542. During incubation of the tumor tissues with the drugs, it was observed very strong effect of 5-fluorouracil, significant but partial response to oxaliplatin and gemcitabine. Iressa and SB431542 both showed an cell-killing effect, but the response was partial (Fig. 6, *A*). In parallel, Functional Biochemical Assays were applied to the tumor samples. These tests showed significantly enhanced activity of pro-mitogenic kinase Erk1/2, as compared to the Erk1/2 activity in the control carcinoma cells (Fig. 6, *B*). Other biochemical tests showed that EGF and TGFβ signaling pathways were active, but at the levels comparable to the control carcinoma cells. The biochemical tests indicate that inhibitors of Erk1/2 pathway, e.g. MEK1 or Raf inhibitors, may have a strong inhibitory impact on the tumor growth for this patient. The tests confirmed also that Iressa and SB431542 indeed may have tumor-suppressing effect. Thus, the Organ Culture FMDx and biochemical tests provided the oncologist with information about efficacy of the tested drug, and indicated that the combination of chemotherapy with adjuvant therapy may be beneficial for the patient.

Organ Culture FMDx delivers informative results during the first 2 weeks of culturing. Longer culturing leads to changes in structure of the tumor tissue, and changes in physiology of tumor cells. These changes affect pattern of the cell response to the drugs. However, longer culturing allows obtaining primary culture of tumor cells which detach from the tumor tissue. These primary culture cells may be further used for research purposes. For this patient, a culture of primary cells was obtained. Thus, Organ Culture FMDx delivered information about sensitivity of the tumors of the patient to the selected drugs.

Case 3. Generation of individualized cancer vaccine

There is a strong confidence among oncologists that as long as the vital functions of a body are not compromised, there is a chance of curing even advanced cancer. One of the curation strategies is deciphering of the molecular profile of the tumor, and finding combination of drugs which may kill this tumor. These strategies are mentioned in descriptions of the cases #1 and #2. The case #3 describes the strategy to employ immunological protective mechanisms of the patient's body.

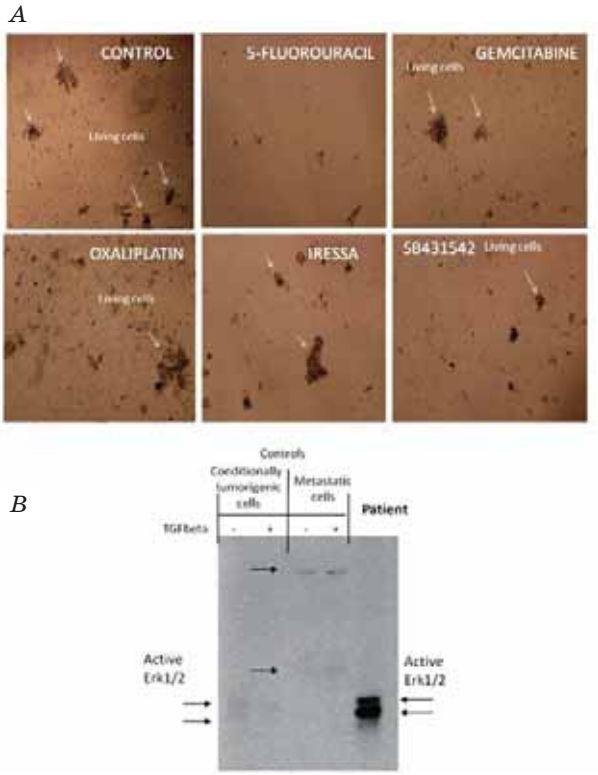

Fig. 6. **Organ Culture FMDx**

Organ Cultures are prepared from the tumor biopsy. *A* — Images of the organ culture cultured in a medium only (Control), or incubated with drugs as indicated. Arrows show clusters of living cells. Organ cultures were incubated with the drugs for 48 h. Note presence of living cells in cultures incubated with gemcitabine, oxaliplatin, Iressa and SB431542.

B —Functional Biochemical Assay with the same tumor samples as in (*A*). The assay was to evaluate activity of Erk1/2 kinase. Note strongly enhanced Erk1/2 signal in the tumor sample, as compared to conditionally tumorigenic and the metastatic control samples. Note that the Erk1/2 signal in the tumor corresponds to the Erk1/2 characteristic in the non-aggressive tumor cells. This assay indicates that the drugs inhibiting Erk1/2 may be beneficial for this patient.

The idea of the individualized cancer vaccine is based on the fact that the body is capable to recognize the tumor epitopes, but the response is too weak to remove the tumor. Therefore, boosting of the anti-tumor immunity is required. Currently, the first type of the anti-tumor vaccines is based on identification of tumor-recognizing lymphocytes, which are then expanded in vitro, and injected in the patient. The second type of vaccines is based on identification of tumor antigens which stimulated immunological response, even though weak and not sufficient to eliminate the tumor. These antigens are then purified, and used to boost the anti-tumor immune response.

The presented here case is an example of how such an antigen-based vaccine could be developed (Fig. 7). The patient described in this case could not be subjected to biopsy, due to the weak general condition and numerous metastases. Therefore, a blood sample was taken, and plasma was prepared. The plasma was used to detect tumor-specific antigens. The antigen array was prepared by 2D-GE, and transferring of separated proteins from the gels on the membranes. The transferred tumor proteins were probed with the patient's plasma, and with plasma of healthy individuals. To discriminate tumor and non-tumor antigens further, protein arrays from the non-tumor samples were prepared, and probed with plasma from the patient and from healthy individuals. All these tests allowed identification of 2 strong tumor-specific antigens (Fig. 7, *A*). These antigens were then tested as a scratch-test on the patient. As expected, the antigens showed immunological reactions, with one of the antigens showing reaction justifying use of this antigen for development of the individualized vaccine (Fig. 7, *B*). This selected antigen was prepared in quantities required for vaccination. The antigens were purified, and were subjected to tests of chemical purity, toxicity and sterility. The last test before application of such a vaccine is the scratch-test of responsiveness on the patient. The result was considered positive when there was observed a swelling and redness response after 2 days. Thus, application of proteomics allows generation of truly individual cancer vaccine.

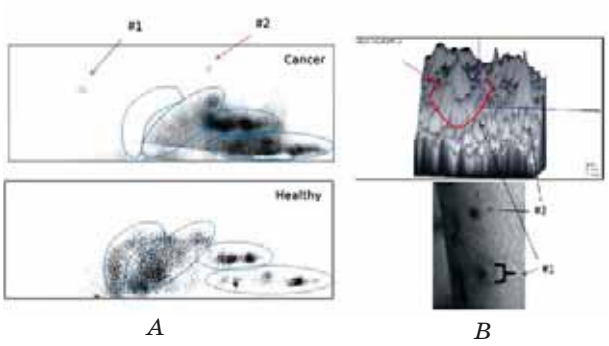

Fig. 7. **Development of individualized vaccine as part of FMDx:**

A — Proteomics is an important part in identification of tumor-specific antigens. Upper panel shows proteins separated in a 2D gel, and recognized by the antibodies of the patient. Lower panel shows the same proteins immunoblotted with antibodies from a healthy individual. #1 and #2 indicate proteins recognized as immunogenic tumor-related antigens.

B — Identified immunogenic tumor-related antigens were purified, and prepared as a vaccine. The scratch test with the vaccine is shown. The scratch test is performed before applying the vaccine, as part of controls. Note that the antigen #1 produced immunological response. The area of the skin is shown in the lower part of the image, and the computer-assisted analysis of the swelling is indicated in the upper part as the area marked by the red line.

Prospective

Complexity of cancer requires comprehensive evaluation of tumors and patients. Therefore, OMICs technologies have come to stay. We may expect significant improvements in quality of results delivered by profiling of genome, transcriptome, proteome and metabolome of a cancer patient and her/his tumors. Cost efficiency of OMICs technologies will allow performing such profiling on every patient. As an example, FMDx is already affordable for routine use in the cancer clinics.

Developments of OMICs technologies are promoted by the combination of biochemistry, cell and molecular biology, engineering, physics, chemistry and mathematics. When in 1961 Yurij Gagarin opened the era of manned space flights that was the result of collaboration between many different professionals. The same is valid for cancer — to make a breakthrough and find cure of cancer, a single discovery is not enough. It has to be a combined effort. And the biggest challenge is to create such an organization which would focus on fighting cancer with understanding complexity of this disease and hosting diverse expertise.

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ІНДИВІДУАЛЬНИЙ ПІДХІД У ЛІКУВАННІ РАКУ: ВНЕСОК OMIC-ТЕХНОЛОГІЙ У ДІАГНОСТИКУ ЗАХВОРЮВАННЯ

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Рак — хвороба, яка є показовою ілюстрацією успіхів, невдач і перспектив сучасних біомедичних досліджень. Розвиток відповідних технологій став вирішальним чинником поліпшення якості лікування раку. Впровадження в клінічну практику досягнень технологій геноміки, РНК-профілювання та протеоміки забезпечило основу для розроблення нових діагностичних засобів, лікарських препаратів і методів лікування.

У статті обговорено внесок OMIC-технологій у персоналізацію діагностики та лікування цього захворювання. Акцент зроблено на технологіях, які показали можливість відповідного доставлення діагностичних засобів, що може бути використано в клініці для простих діагностичних тестів. Як ілюстрацію наведено три клінічних випадки з використанням доступних на сьогодні методів персоналізованої діагностики раку.

Ключові слова: персоналізоване лікування раку, діагностика, геноміка, транскриптоміка, протеоміка, метаболоміка.

ИНДИВИДУАЛЬНЫЙ ПОДХОД В ЛЕЧЕНИИ РАКА: ВКЛАД OMIC-ТЕХНОЛОГИЙ В ДИАГНОСТИКУ ЗАБОЛЕВАНИЯ

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Рак — болезнь, которая является показательной иллюстрацией успехов, неудач и перспектив современных биомедицинских исследований. Развитие соответствующих технологий стало решающим фактором улучшения качества лечения рака. Внедрение в клиническую практику достижений технологий геномики, РНК-профилирования и протеомики обеспечило основу для разработки новых диагностических средств, лекарственных препаратов и методов лечения.

В статье обсуждается вклад OMIC-технологий в персонализацию диагностики и лечения этого заболевания. Акцент сделан на технологиях, показавших возможность доставки диагностических средств, что может быть использовано в клинике для простых диагностических тестов. В качестве иллюстрации приведены три клинических случая с использованием доступных на сегодняшний день методов персонализированной диагностики рака.

Ключевые слова: персонализированное лечение рака, диагностика, геномика, транскриптомика, протеомика, метаболомика.

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PLANT TISSUE CULTURE IN BIOTECHNOLOGY: RECENT ADVANCES IN TRANSFORMATION THROUGH SOMATIC EMBRYOGENESIS

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Plant genetic transformation has become an important biotechnology tool for the improvement of many crops. A solid foundation for the fast development and implementation of biotechnology in agriculture was provided by achievements in plant tissue culture. On the 30th anniversary of plant transformation, I report the advancements, recent challenges and shifts in methodology of transformation. The main focus of this paper will be on conventional and novel approaches for genetic improvements of soybean, cotton and corn. I will also highlight results on the transformation of these crops that have considerably been improved by modern biotechnology.

Key words: plant tissue culture, transformation, genetic improvement of crops.

The development of cell, tissue and organ culture methods was rapidly accelerated in the second half of the previous century after establishing robust cell culture techniques and media formulation for *in vitro* growth of plant material [1–8]. A stunning number of articles have been published on *in vitro* induction and maintenance of non-differentiated cells and the regeneration the plants from them whether through organogenesis or somatic embryogenesis. Fine protocols were established for the culture of enzymatically isolated single cells and protoplasts [9], which were able to regenerate into plants [10–12]. *In vitro* culture methods became an essential part of many micropropagation protocols. Culture of plant cells and organs in bioreactors were used for the production of different secondary metabolites and pharmaceuticals. *In vitro* techniques were used for production of mutants, haploids, virus-free material, and also for maintenance and preservation of rare genotypes and specific cell cultures [13–15].

The first positive results on *Agrobacterium*-mediated transformation in plants were reported in 1983 [16–18]. A simple method for transferring genes into plants through the inoculation of leaf discs with *Agrobacterium tumefaciens* followed by *in vitro* culture and regeneration of whole plants was reported by Horsch et al. [19]. Different methods for DNA delivery into plant cell, including electroporation [20], PEG treatment [21, 22], microinjection [23], sonication [24], biolistics or particle bombardment [25], silicon carbide Whiskers™ treatment [26, 27], were used for specific transformation purposes and different types of cells and genotypes. However, *Agrobacterium*-mediated transformation became a preferred method. *Agrobacterium tumefaciens* is a natural vector system for transgenes delivery into a wide range of plants species, providing an efficient and «clean» insertion of DNA into the plant genome and deserved to be called the «tzar of genetic engineering» [28]. In the past 30 years the discovery and application of new transformation technologies essentially sped up the improvement of major cultivated crops. The first, really commercially grown plants hit the market in the mid — 1990s.

Practically all transformation systems were based on *in vitro* culture methods. It was impossible to transform whole plant organism at once. All techniques were based on transforming single cells of callus, leaves, pollen, roots or other organs and than regeneration of plants through somatic embryogenesis or organogenesis. Routine and highly efficient transformation methods for many important crop and particular genotypes were implemented in many biotechnology companies.

However, laborious and time consuming *in vitro* methods of transformation limited the production of transgenic events. The biotechnology industry requires simple, high throughput, genotype independent transformation systems, which also could be marker- and selection-free. Whole plants, seeds, mature embryos, flowers, meristems, stolons and other plant organs became a target for transformation with a purpose to produce transgenic plants without using *in vitro* methods. The application of meristematic cultures has become a valuable tool for transformation of some recalcitrant species since it is less genotype dependant and due to possible short culture period had less potential problems with somaclonal variation [29].

In this report, as a tribute to cell biologists and their huge impact in development of modern biotechnology, the overview of conventional techniques used for transformation of important crop plants and some advancement in this area will be presented. Comprehensive reviews on molecular aspects of transformation and on milestones in plant tissue culture can be found elsewhere [30, 31]. Herein the information on transformation will be updated with emphasize on transformation through embryogenesis in some legumes, woody plants and cereals which are commonly considered as recalcitrant.

Soybean transformation

Roundup Ready® soybean developed by Monsanto was one of the first transgenic crop commercialized in 1996. For the development of this new product a bacterial gene for a glyphosate-tolerant variant of EPSP syntase (CP4) [32] was transferred, by particle bombardment, into embryonic axes of excised soybean embryos, which were regenerated into plants by organogenesis [33]. Further improvements in soybean transformation were with the development of *Agrobacterium*-mediated transformation of cotyledon explants, which also underwent organogenesis [34]. Considerable advancement in soybean transformation was observed after development of new high throughput technology based on *Agrobacterium*-mediated transformation of excised mature embryos [35, 36]. In the meantime a lot of research was concentrated on the development of embryogenic culture transformation and/or regeneration of plants through somatic embryogenesis [37, 38]. First efficient induction of embryogenic culture from immature cotyledons was described by Lippmann and Lippmann [39]. The most reproducible particle bombardment transformation system was based on soybean embryogenic culture protocols which came from the labs of Finer and Parrott [40–42]. Soybean embryogenic cultures were also transformed by using *Agrobacterium* [43, 44] but this method turned out to be less efficient and not always reproducible.

Although embryogenic cultures of soybean are not the best target for transformation some biotechnology laboratories and companies are still using it for commercial production of transgenic plants. Since it was demonstrated that somatic embryos could be comparable to seeds in terms of quantity and especially quality of oil and protein [45–47], transformation of embryogenic cultures and production of transgenic soybean somatic embryos has been used in assays for rapid analysis of seed traits [48]. To some extend the wide application of embryogenic culture in transformation was limited due to genotype dependence. The cultivar Jack has given the best *in vitro* response, however, other genotypes can also be used for induction of embryogenic culture but with lower efficiency.

For the initiation of somatic embryos from immature cotyledons usually MSD40 medium containing 40 mg/l 2,4-D [49] is used. MSD20, which is the same medium as MSD40 but with 20 mg/l 2,4-D, is used for maintenance of embryogenic culture. For liquid culture the FN Lite medium [50] with 10 mg/l 2,4-D plus 0.5 mg/l Picl (or 0.1 mg/l Kin) is recommended. Embryogenic cultures on MSD20 are more developed while on FN Lite the culture is more globular and much greener. Established embryogenic cultures are usually transformed by biolistic methods. Detailed condition and media for induction of culture and transformation with biolistics can be found in different publications [41, 51, 52]. For selection of transgenic embryo-cultures hygromycin was mainly used.

At Monsanto our research confirmed the feasibility of using embryogenic cultures for transformation and developed a transformation system with *NPTII* as selectable marker. Embryogenic cultures were established from immature cotyledons of Jack and other genotypes. The scheme for isolation of immature cotyledons from pods and the induction of somatic embryos is shown on Fig. 1, *A*. Initiation of somatic embryos from immature cotyledons and the morphology of callus on MS40 and FN Lite medium can be seen on Fig. 1, *B, C, D*.

Efficient delivery of foreign DNA (*NPTII*/GFP construct) was established by particle bombardment. As a target for bombardment we

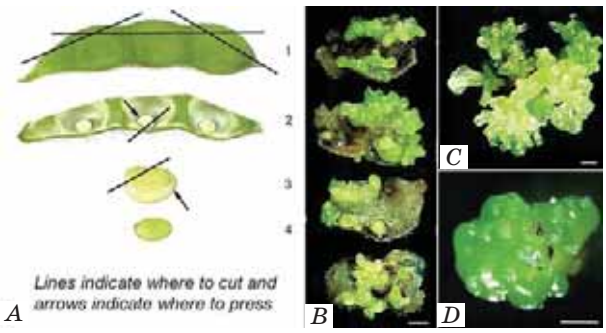

Fig. 1. **Scheme for isolation of immature cotyledons from a soybean pod (*A*), induction of somatic embryos on MS40 medium (*B*) and embryogenic callus on MS20 and FN Lite media (*C, D*). Bar: 1 mm**

used a young embryogenic callus grown in a dim light on MS20 medium. In spite of efficient DNA delivery into cells (high transient GFP expression was observed after 24 h of culture, Fig. 2, *A*) the recovery of stable transformants was very low. Several antibiotics and different selection pressure were tested for selection. It was found that kanamycin even at concentration of 200 mg/l did not bleach and inhibit the growth of green embryogenic cultures. Suitable for selection was paromomycin with an optimal concentration 50 mg/l. First stable transgenic events were usually identified after 3 weeks of selection (Fig. 2, *B*). Selected callus was propagated in the presence of paromomycin and than transferred to a new medium for embryogenesis (Fig. 2, *C, D*). Very fast embryogenesis and embryo maturation was established on SHAM medium (modification of FNL0S3 [52]). The embryos after 9 days of culture on this medium are shown (in a blue and day light) on Fig. 2, *D, E*. After maturation the developed embryos were germinated on SHAM or MS media without plant growth regulators (PGR).

In general, the low transformation frequency (TF) of soybean embryogenic cultures, observed in our work and in other reports, is probably due to the origin of the transformation target which is highly developed multicellular somatic embryos, and their sensitivity to selection agents. Even in spite of repetitive formation of somatic embryos observed in *in vitro* cultures, the chances for recovery of transgenic events are low. Due to development of novel and extremely robust alternative transformation techniques it is difficult to see a wide application of soybean embryogenic cultures in commercial production of genetically modified soybean plants although it is still very valuable for fundamental research and for development of different transformation assays.

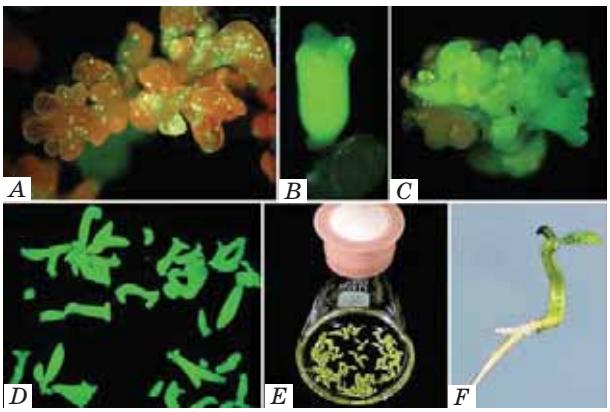

Fig. 2. A **— Transient GFP expression in soybean embryogenic callus (1 day after bombardment with NPTII/GFP construct). *B, C, D* — GFP expression in single somatic embryo, callus and mature somatic embryos obtained from embryogenic callus after 9 days of culture on SHAM medium. *E, F* — Maturation of somatic embryos in suspension culture and their germination.**

Cotton transformation

Cotton is another economically important agricultural crop which is transformed by *Agrobacterium* or particle bombardment and mainly regenerated via somatic embryogenesis. Insect resistant, Bollgard® cotton, which was first transformed in 1987 [53, 54], was commercially released in 1996. Cotton is woody dicotyledonous plant, and it is truly recalcitrant to *in vitro* regeneration with very strict genotype dependence. Since the first reports on transformation, successful regeneration via embryogenesis is mainly limited to cotton varieties with a Coker pedigree. Although regeneration of cotton via organogenesis has been reported [55] there are only a few available publications on transformation and regeneration through organogenesis [56, 57]. *Agrobacterium*-mediated transformation of Coker genotype with regeneration by means of somatic embryogenesis was the most efficient method for generating transgenic cotton plants [58, 59]. This method, however, is labor-intensive and time-consuming. *Agrobacterium*-mediated transformation system *via* embryogenesis usually requires a period of up to twelve months for production of transgenic cotton plants and in comparison to other crops is rather inefficient. Regeneration and transformation methods were also established for other cotton genotypes [60–63] and, in spite of low efficiencies, were utilized for specific needs.

Other methods, like particle bombardment [64, 65] or Whiskers™-mediated transformation [66], have been exploited for the transfor-

mation of embryogenic cotton suspension cultures. There are also a few reports on pollen and pollen-tube-pathway transformations [67, 68]. In several protocols transformation of the meristem in the shoot apex with particle bombardment or *Agrobacterium* was used [69–71]. A similar approach was applied by Balasubramani et al. [56] who conducted *Agrobacterium* transformation of the embryonic axis of germinated seeds. In these intact plant tissue cases the transformation is rapid and genotype-independent. Since there is no callus stage and practically no cell dedifferentiation (it is based on multiple shoot formation) the chances of somaclonal variation are low. At the same time very light selection pressure used for meristem transformation can often be associated with chimerism of the produced shoots and plants [59]. No doubts that such methods with further improvements will be used as the foundation and essence for new industrial technologies.

Here it will be summarized the improvements made at Monsanto on the conventional transformation of Coker 130 genotype through embryogenesis which were presented earlier [72]. This protocol is similar to others and includes several steps: 1) *Agrobacterium tumefaciens* inoculation of hypocotyls cuttings and co-culture; 2) Induction of «creamy and soft» friable, undifferentiated callus; 3) Initiation and selection of embryogenic callus (EC); 4) Embryo maturation and germination; 5) Transplanting into soil. We developed and implemented a liquid-based culture system that drastically increases the efficiency of plant production. For protocol development *Agrobacterium tumefaciens*, a modified C58 strain of the bacterium with *NPTII*/GFP and *NPTII*/GUS constructs and kanamycin selection (70 mg/l) were used. Some production steps of transgenic cotton are illustrated on Fig. 3.

Callus induction was performed in plates with liquid medium. With a new culture system we could speed up the production of EC and eliminate multiple sub-culturing. Higher concentration of gelling agent in the regeneration medium especially covered with nylon «mesh» (100% nylon organza fabric) provided faster conversion of embryogenic callus and maturation of embryos (Fig. 3, *F, G, H*). Overall, with an optimized liquid transformation system the time frame for plant production could be cut in half.

Further improvements were connected with the development of *Agrobacterium*-mediated transformation system based on using EC as an initial explant. The initial EC material was easy to maintain on medium without growth regulators and could be transformed with *Agrobacterium* only if EC was desiccated during co-culture with the bacteria. Earlier it was demonstrated that desiccation during co-culture enhances the T-DNA delivery into plant cells and could be critical for transformation of callus [74]. Using GFP and GUS as reporter marker it was shown that transgenic plants could be produced in about 3 months after transformation (Fig. 4).

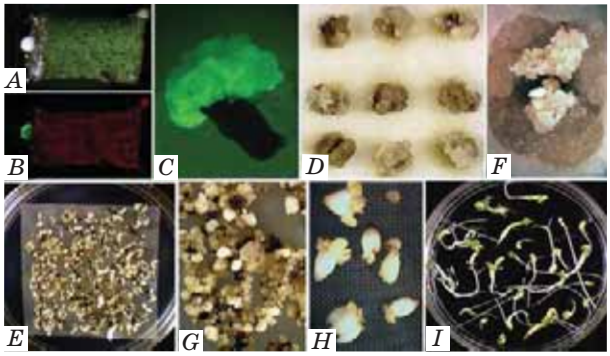

Fig. 3. **Different stages of cotton hypocotyls transformation:**
A-E — Transgenic callus formation in liquid WPSEL medium which contains Lloyd an McCown salts according to Phytotechnology Labs, 2 ml/l Gamborgs B5 vitamins (500x, Phytotechnology Labs, 0.1 mg/l 2,4-D, 0.5 mg/l kinetin, 30 g/l glucose, pH 5.8. *A, B* — 7 d old callus under day and blue light; *C* — GFP expression in 1 month old callus; *D* — 1 month old callus, cultured in a liquid medium; *E* — EC formation after 3 month of selection; *F, G* — Formation of globular embryos on TRP medium (MS salts and 2 ml/l Gamborg B5 vitamins according to Phytotechnology Labs, 0.1 g/l casein hydrolysate, 30 g/l glucose, 2.5 g/l Phytagel®, pH 5.8); *H* — Embryo maturation on TRP medium with 7 g/l agarose; *I* — Embryo germination on ShSu medium (Stewart and Hsu salts and vitamins [73], 5 g/l sucrose, 2.5 g/l Phytagel, pH 6.8).

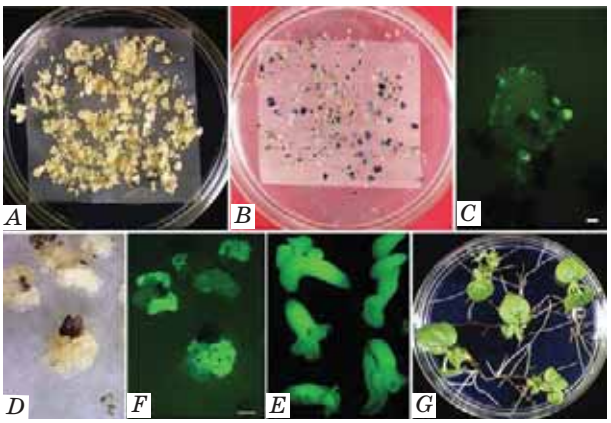

Fig. 4. **Agrobacterium-mediated transformation of embryogenic callus:**
A — Friable EC used for transformation; *B* — Transient GUS expression in embryogenic callus; *C* — GFP expression after 14 days of selection; *D, E* — Callus culture under blue and day light after 1.5 month; *F* — GFP expression in somatic embryos during maturation after 2.5 months; *G* — Plantlet formation from somatic embryos after their maturation. Bar: 1 mm

Corn transformation

Corn is one of the most economically important crops which was considerably improved by modern biotechnology. Although the first positive results on the transformation of corn and regeneration of transgenic plants were reported in 1990 [75, 76] only in 1997 did Monsanto Company bring to the marketplace the GMO plant, insect-protected corn in the form of YieldGard® Corn Borer. Similar products were developed by other companies. In 1996 Ciba-Geigy and Mycogen introduced E-176 corn and in 1997 Novartis introduced Bt-11corn. Particle bombardment of corn embryogenic cultures was used for producing the first product. Later not only single traits but stacked traits were added to corn products. To create biotech products two transformation techniques for transgenes delivery have mainly been employed: *Agrobacterium*-mediated transformation [77–80] and particle bombardment transformation [81, 82]. Although corn, like other cereals, is not a natural host for *Agrobacterium* sp., *Agrobacterium*-based transformation is the preferred method and has several advantages over biolistics. New *Agrobacterium*-transformation methods are very efficient and in comparison to bombardment provide a higher number of events with single intact transgenes. Now new products like Genuity™ SmartStax™ (Monsanto), Herculex® Xtra Roundup® Corn 2 (Dow Agrosciences and Pioneer Hi-Bred), Agrisure® GT/CB/LL (Syngenta), Optimum® AcreMax® Insect Protection (Pioneer Hi-Bred, DuPont) and many other have combinations of stacked traits against different insects and tolerance to a number of herbicides for broad spectrum weed control. In 2011 twelve countries have planted biotech crops with two or more traits.

Type I culture in transformation. From the very first publication on corn *in vitro* regeneration [83] it was accepted that somatic embryogenesis is the principal way of plant regeneration in corn and other cereals. The induction of so-called «Type I» callus was established from immature embryos (IE) of inbred line A188. The same compact, organized «embryogenic» type of callus was induced from IEs of number genotypes [84–88]. Right now in most transformation protocols the plants are regenerated through Type I culture. The transformation of IEs and regeneration through somatic embryogenesis or organogenesis is the most popular choice for transgenic plant production. The wide application of *Agrobacterium*-mediated transformation of monocotyledonous species and particularly corn became feasible after development of an efficient transformation method for rice described by Hiei et al. [89]. The disarmed *Agrobacterium,* which was induced by acetosyringone and carried a «super binary» vector with selectable marker genes, was used to establish a corn transformation protocol with freshly isolated IEs [78, 80, 90, 91]. After considerable improvement *Agrobacterium*-mediated transformation of freshly isolated or precultured IEs became a routine practice for efficient production of transgenic corn [80, 92]. However, some corn genotypes possess a high competence for embryogenesis and regeneration but they are not susceptible to *Agrobacterium*. In Fig. 5 a high level of GUS and GFP transient expression in IEs of proprietary genotype, L1 is shown (Fig. 5, *B, C*), and poor transient GUS expression in IEs of another tested proprietary genotype L2 (Fig. 5, *A*).

In order to obtain a high TF with «difficult» genotype it could be necessary to do an essential protocol modification and media optimization. Very often it can be easier to screen the breeders potential candidate-genotypes for «culturability» (competent for *in vitro* culture and regeneration) and «transformability» (competent for transformation) using several standard protocols. Sometimes it could be a good idea to test for transformation of other explants. Thus, one genotype which was difficult to transform using freshly isolated IEs (Fig. 5, *A*) turned to be highly transformable when young callus of cultured IEs were used as initial explants for transformation (Fig. 5, *D*).

High TF can be achieved with freshly isolated IEs or propagated *in vitro* callus obtained from IEs or seedlings [80, 93]. Because of potential somaclonal variation, extended maintenance of callus *in vitro* is not recommended. Production of seedling-derived callus using mature seeds has a high impact on trans-

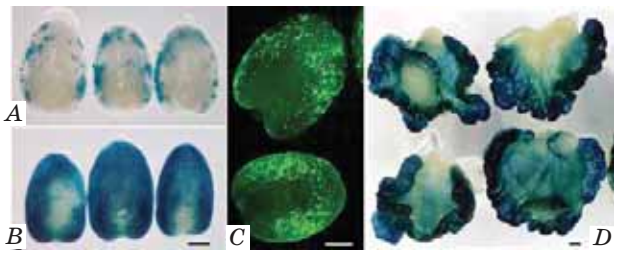

Fig. 5. **Transient GUS and GFP expression in freshly isolated (*A, B, C* different genotypes) and transient GUS expression in callus cells after culturing of IEs for 8 days. GUS staining for 3 hours.** Bar = 0.5 mm

formation technology since it is considered as «greenhouse-independent» (it does not require growing plants for IEs). Transformation technology based on using seedling-derived callus can be efficiently utilized in dihaploid programs and potentially speed up the breeding process. Haploid Type I callus can be produced from seedlings of haploid seeds conventionally obtained after crossing a corn variety with a haploid inducer line. Haploid seeds are easy to identify due to the presence in the inducer line of visible pumule and cap markers. Routinely about 75% of haploid callus lines from seedlings remain haploid. Isolated haploid callus can be transformed and after doubling homozygote dihaploid transformed plants can be regenerated [94, 95].

Different selection scheme can be used for preferential growth of transformed cells and regeneration of transgenic plants. For commercial production of transgenic plant genes conferring resistance to antibiotics, herbicides and mannose have been used [96]. Selectable marker genes can be removed since they are not required for expression of the gene of interest. There are several methods available for marker removal from transgenic plants: co-transformation with 2 unlinked T-DNAs («2T transformation») followed by segregation of the marker gene in progeny [97-99], homologous recombination between direct repeats [100] and site-specific recombination, including the most popular Cre/*lox* system [101, 102]. Extremely appealing is marker-free, selection-free transformation technology [103, 104].

Only with very high TF it is possible to do the transformation without selection and conduct identification of transgenics by molecular screening of all produced shoots or plants. Since we developed a very efficient IEs transformation protocol for L1 line with TF in some experiments higher than 60% we checked the feasibility of using no selection for production transgenic plants.

Using ABI *Agrobacterium* containing a construct with the *uidA* (GUS) reporter gene we tried to track the formation of stable transgenic events during different stages of culture and evaluate the efficiency of transformation without selection. After 10 days of culture post- transformation with no selection it was found that almost all IEs formed a callus with several GUS positive regions. After several weeks of culture the callus derived from each IE was divided into 10–20 pieces and cultured further (Fig. 6). It was found that all callus pieces from a single IE were GUS negative, a few were chimeric and one was non-chimeric, GUS positive. A similar picture was observed in several other cases when the batch of callus pieces derived from single IE were stained. Among plants regenerated without selection we also identified several GUS positive (Fig. 6, *D*). Although in our experiments the TF was very low this method after improvements of screening at the callus stage can be useful. Such a marker-free/selection-free protocol allows doing the transformation of corn with 1T constructs without any additional markers and in comparison to transformation with 2T constructs it reduces the breeding timeframe and simplifies the downstream breeding process.

Type II culture in transformation. A new type of embryogenic callus named as «Type II»was described by Green [105, 106] and Armstrong, Green [107]. Type II culture derived from immature embryos is a very fine, friable callus which can directly form somatic embryos. Efficient induction and sustainable

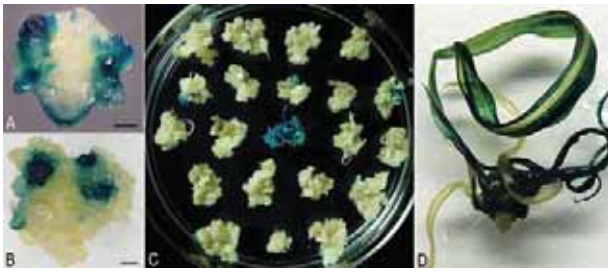

Fig. 6. **GUS expression in callus derived from IEs on different stages of culture and regeneration (without selection) after transformation with GUS construct:**
A, B — Transient GUS expression in IEs callus after 6 days and 14 days of culture; *C* — Callus pieces on regeneration medium; one transformant and few himeric clumps were identified among callus pieces originated from one IE; *D* — GUS positive plantlets from no-selection experiment. Bar: 1 mm

propagation of embryognic callus was established with A188 inbred genotype on N6 [7] medium supplemented with 6 mM proline [107]. The induction of this type of callus is highly genotype dependent. It was demonstrated that many hybrids which have A188 as one parent also demonstrate the embryogenic response found in A188. The well known genotype with Type II response is «Hi-II» derived from progeny of A188×B73 cross [108]. Quantitative trait loci (QTLs) that control regenerable callus formation and plant regeneration in maize have been identified for the Type II callus response [109, 110]. Embryogenic Type II callus was usually initiated from

IEs but the induction of such culture from immature tassels of HiII genotype has also been reported [111]. The Hi-II line is not an inbred but Lowe et al. [112], after crossing Hi-II with the FBLL genotype followed by multiple backcrosses transferred the Type II embryogenic response developing the FBLL-MAB inbred line. The availability of suitable inbred lines for transformation is particularly desirable since modern breeding of corn is based on the utilization of inbred parents for production of hybrid seeds possessing «hybrid vigor» or heterosis. It is typical that breeding/biotechnology companies have their own proprietary germplasm that are bred for traits like yield or disease resistance not for *in vitro* «culturability» and «transformability». Some of these elite genotypes require, for *in vitro* growth, completely new combinations of PGR in media for them to be transformed with high efficiency. It was demonstrated that Type II callus is not limited to A188 genotypes or close relatives and can be obtained from different genotypes [113, 114]. Type II callus can be directly used for transformation [76] and can be a good source for protoplast isolation and production of transgenic plants [115]. Still, genotype specificity is much higher for Type II callus than for Type I callus. Due to the high genotype dependence the regeneration through somatic embryogenesis from Type II callus has been used less and less in the commercial transformation of corn.

Meristem culture in transformation. Corn and other cereals can also be regenerated via organogenesis. First multiple shoot formation from apical meristem of immature embryos was reported in 1992 [116]. Induction of organogenic cultures from corn seedling meristem was described by Zhong et al. [117, 118]. The same type of culture was initiated from meristematic tissue of the nodal area of seedlings, leaf bases of young leaves and other explants containing highly meristematic cells. The culture medium for multiple shoot induction usually contains high concentration of cytokinins (0.5–10 mg/l 6BA) and no or low concentration of auxins (0–0.5 mg/l 2,4-D). The same or similar types of media were used for multiple shoot induction in oat [119], sorghum [120], millet [121], wheat [122], barley [123] and other monocots. Meristem culture depending on the stage of development may have multiple buds resembling shoot apex with apical meristem and primordial leaf, enlarged buds, highly meristematic zones with multiple buds or more developed multiple shoots (Fig. 7). Meristem shoot culture can be maintained for

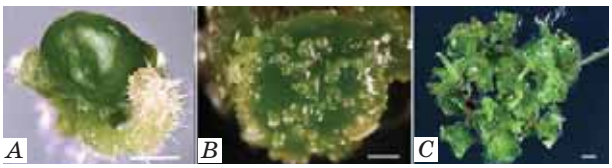

Fig. 7. **Different development stages of corn shoot meristem culture:**
A — Enlarged bud; *B* — Adventitious buds formation from highly meristematic tissue; *C* — Established meristem shoot culture. Bar: 2 mm

extended period of time and can easily produce plants on the medium without PGR [124].

Shoot apical meristems and derived organogenic cultures were recommended for use as sustainable explants for genetic transformation of cereal crops [119]. This organogenic type of culture, referred to as apical meristem culture, shoot meristematic culture, multiple shoot culture, and multiple bud culture, has been transformed by particle bombardment [125, 126]. Induction of this type of tissue appears is less dependent on specific genotype in comparison to Type II and Type I cultures [119]. For wide implementation of organogenic culture in production work it still requires the development of efficient delivery of foreign DNA via *Agrobacterium*.

General remarks on corn morphogenesis. The morphology, characteristics of Type I, Type II, and meristem culture with the possible conversion pathway of one type culture to another are presented on Fig. 8. Depending on the genotype the conversion of one type of culture to another can be very fast but often requires several subcultures. Conversion of organogenic meristem culture of LH198 x HiII and L1 line into Type I callus is presented below (Fig. 9, *A, B*). Conversion of FBLL-MAB Type II callus into shoot meristem culture is shown on Fig. 9, *C, D, E*.

In scientific lexicon Type I culture is commonly described as «embryogenic» which is not completely correct. It is known that term somatic embryogenesis describes a developmental process of somatic cells which results in a morphological structures similar to zygotic embryos and typically had a distinct developmental stages. In comparison to Type II callus which is truly embryogenic, Type I callus is not. It is more organogenic since at light condition it is easy converts into leafy structures and forms shoots (Fig. 10). Detailed histochemical and ultrastructural study show that Type II callus does contains embryogenic units (which give rise to somatic embryos) while Type I callus has mainly a meristematic cells and extensive vascular network [127, 128].

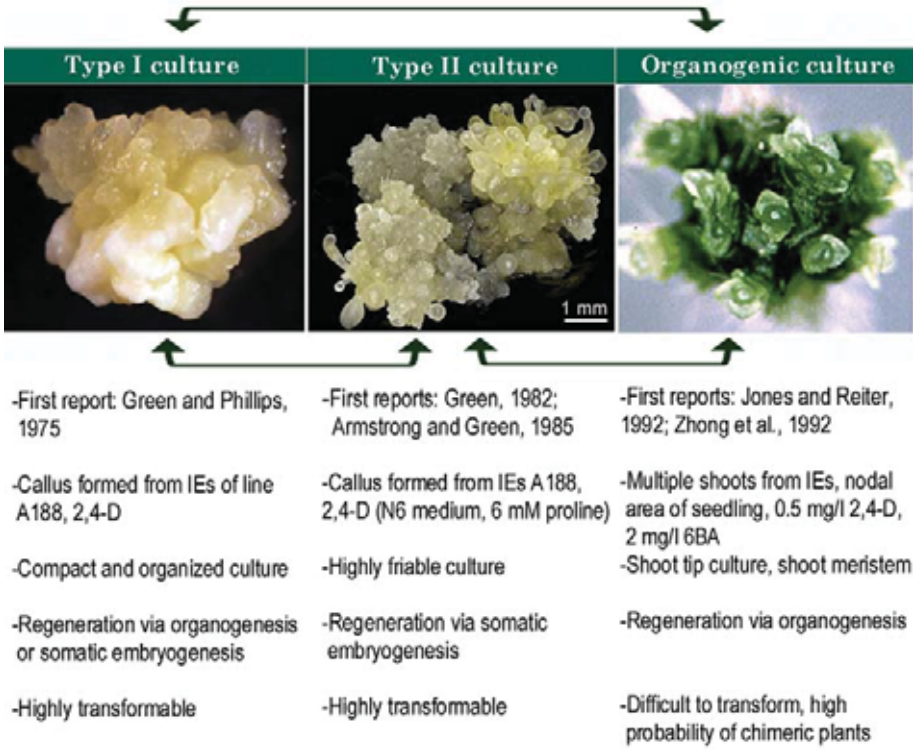

Fig. 8. **Regenerable types of in vitro corn culture, their main characteristics, and conversion pathway of one type culture to another**

Fig. 9. **Conversion of organogenic meristem culture of LH198 x HiII (*A*) and L1 line (*B*) into Type I callus. Bar: 2 mm. *C, D, E* — Conversion of FBLL-MAB Type II callus into meristem culture with multiple buds; 2 and 4 weeks culture on MSV34 medium [80]. Bar: 0.9 mm**

Greening of this type of callus, formation of leafy structures, followed by multiple buds and shoot formation are especially pronounced in production experiments where for a fast regeneration the callus is exposed for a short time to 6BA (MSBA medium according to [80]). As it is shown on Fig. 11, Type I callus

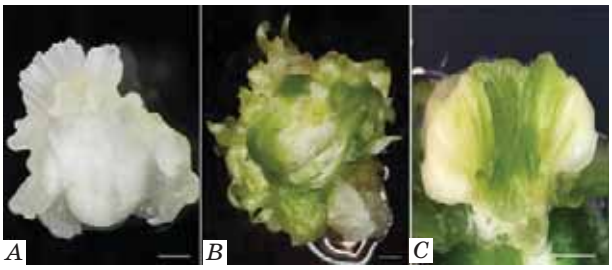

Fig. 10. ***A* — Type I callus formation from IE LH198 x HiII (in dark). *B* — The same IE cultured another 10 days on the same medium (on light). *C* — Piece of callus on higher magnification which converts into leafy structures. Bar: 1 mm**

after a short culture in light on 6BA medium formed green leafy structures with multiple buds at the base of them. On PGR-free medium these buds formed shoots which could be later rooted. Formation of multiple buds (apical meristem) from Type I callus in corn is demonstrated on Fig. 11, *A, B*. Because of this regeneration pathway there is no stage of somatic embryogenesis and it is properly to call this way of morphogenesis as organogenesis. At the same time the regeneration of Type I callus through embryogenesis is also possible. In several publications compact Type I callus was regarded as «fused deformed and normal

Fig. 11. A, B — **Multiple buds and shoots formation in corn after 1 week culture on 6BA containing medium, followed by PGR-free medium. *Bar:* 0.5 mm;**
C, D, E **— Germination of corn somatic embryos of L1 line on PGR-free medium;**
C **— Bar: 1 mm;** ***D, E*** **— Bar: 2 mm.**

somatic embryos with an incomplete shoot-root axis» [129, 130]. Biochemical analyses of Type I callus also have shown the presence of specific markers of embryogenesis in this type of callus [131, 132]. It was confirmed that Type I callus on PGR-free medium in darkness can produce very fine callus clumps which in suspension culture could initiate single somatic embryos (Fig. 11, *C, D, E*). Because of presence of two types of cells which regenerate through organogenesis or embryogenesis probably it would be more correct simply to call Type I callus as regenerable callus and only Type II callus refer as embryogenic.

In conclusion, it is expected that the presented results on corn, especially in comparison to the data on other species regenerated through embryogenesis, will clarify some peculiarities of corn culture and transformation. Hopefully this will be useful for researchers working on further improvements of transformation technologies.

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This review is dedicated to Prof. S. Komisarenko on his70th anniversary in honor of his landmark accomplishments in biochemistry and modern biotechnology.

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КУЛЬТУРА ТКАНИН РОСЛИН У БІОТЕХНОЛОГІЇ: ОСТАННІ ДОСЯГНЕННЯ В ГАЛУЗІ ТРАНСФОРМАЦІЇ ЗА ДОПОМОГОЮ СОМАТИЧНОГО ЕМБРІОГЕНЕЗУ

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Генетична трансформація рослин стала важливим інструментом біотехнології для вдосконалення багатьох сільськогосподарських культур. Міцну основу для швидкого розроблення та впровадження біотехнологій у сільському господарстві було закладено досягненнями методу культури тканин рослин. До 30-річчя трансформації рослин у статті описано успіхи, проблеми та останні зміни в методології трансформації. Основну увагу приділено традиційним і новим підходам для генетичного вдосконалення сої, бавовни і кукурудзи. Наведено також результати трансформації цих культур, які були суттєво вдосконалені завдяки здобуткам сучасної біотехнології.

Ключові слова: культура тканин рослин, трансформація, генетичне вдосконалення сільськогосподарських культур.

КУЛЬТУРА ТКАНЕЙ РАСТЕНИЙ В БИОТЕХНОЛОГИИ: ПОСЛЕДНИЕ ДОСТИЖЕНИЯ В ОБЛАСТИ ТРАНСФОРМАЦИИ ПОСРЕДСТВОМ СОМАТИЧЕСКОГО ЭМБРИОГЕНЕЗА

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Генетическая трансформация растений стала важным инструментом биотехнологии для усовершенствования многих сельскохозяйственных культур. Прочная основа для быстрой разработки и внедрения биотехнологий в сельском хозяйстве была заложена достижениями метода культуры тканей растений. К 30-летию трансформации растений в статье описаны успехи, проблемы и последние изменения в методологии трансформации. Основное внимание уделено традиционным и новым подходам для генетического совершенствования сои, хлопка и кукурузы. Представлены также результаты трансформации этих культур, которые были значительно усовершенствованы благодаря достижениям современной биотехнологии.

Ключевые слова: культура тканей растений, трансформация, генетическое усовершенствование сельскохозяйственных культур.

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DEGRADATION OF ANTHROPOGENIC CONTAMINANTS BY HIGHER PLANTS

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Elimination of contaminants from the environment by microorganisms of different taxonomic groups is an evolutionarily determined property, which have already been widely discussed. Until recently, plants still occupying above 40% of the world land, were considered as organisms having only a limited potential for contaminants conjugation and accumulation within cell organelles. Based on 40 years experience in this area author is making an attempts for the evaluation of different aspects of plants ecological potential from the modern understanding; to assume mechanism of inter replacement of enzymes participating in oxidative degradation of organic contaminants in higher plants; to stress the importance of phenoloxidase, enzyme hitherto unknown to participate actively in remediation processes (contaminants oxidative decomposition); to reveal the criterion for the evaluation under the action of contaminants of such precise indicator of plant detoxification potential as deviations in ultrastructural level of plant cells.

Key words: higher plants, anthropogenic contaminants, oxidative degradation, phenoloxidase.

Natural contaminations such as the emission of poisonous gases during a volcanic eruption and earthquakes, swamps poisoned evaporations, synthesis of toxic compounds by lower (microorganisms) and higher plants, etc., in comparison with the human contribution in the environmental contamination is much less impressive. As a result of urbanization, the unpredictable growth of industry and transport, production of chemicals for agriculture, military activities, etc. the concentration of anthropogenic toxicants spread in nature, especially in some regions exceeds all the permissible standards. In spite of difficulties in quantitative, as well as in qualitative estimation, and having a tendency to be increased, the amount of spread out contaminants exceeds annually one billion of tons. Most dangerous among these contaminants are considered as emergent because of their persistence, bioaccumulation, and toxicity along with our awareness of their prominent occurrence in the environment. In different ways, huge amounts of these hazardous substances or toxic intermediates of their incomplete transformations are accumulated in the different niches of biosphere, significantly affecting ecological balance. Lately, many ecological technologies have been elaborated, targeted to minimize the flow of toxic compounds to the biosphere and to control their level or state [1, 2]. Despite the definite positive effect from the realization of these technologies (physical, chemical, mechanical etc), the intensive flow of toxic compounds to the biosphere is still increasing.

Nevertheless, the plants kingdom assimilates toxic compounds, removing them from the environment, naturally providing long-term protection and monitoring against their environmental dispersal. Obviously, microorganisms and plants represent the main power of nature permanently straggling for the maintaining of ecological balance.

Plants being recently recognized as important ecological tool and in order to properly evaluate their detoxification potential; the following ecobiological specificities of these organisms should be emphasized:

– Higher plants simultaneously contact three main ecological niches: soil, water and air.

– Well-developed root system of higher plants determines soil-plant-microbial interaction, representing unique process, significantly affecting the overall plant metabolism.

– Large assimilating surface area of plant leaves (adaxial and abaxial), significantly exceeding in size the above ground surface

under the plant, permits the absorption of contaminants in a big quantity from air via the cuticle and stomata.

– Unique internal transportation system in both directions, distributing all penetrated compounds throughout the entire plant.

– Autonomous synthesis of vitally important organics and extra energy during prolonged remediation process.

– Existence of enzymes catalysing oxidation, reduction, hydrolysis, conjugation and other reactions of multistage detoxification process.

– Large intracellular space to deposit heavy metals and conjugates of organic contaminants.

– Functionalization and further transformation of organic contaminants in plant cells (conjugation, deep oxidation, etc.).

The contaminants penetration into the roots essentially differs from the leaves. Substances pass into roots only through cuticle-free unsuberized cell walls. Therefore, roots absorb substances much less selectively than leaves. Roots absorb environmental contaminants in two phases: in the first fast phase, substances diffuse from the surrounding medium into the root; in the second they gradually distribute and accumulate in the tissues. The intensity of the contaminants absorption process, characterized by various regulations, depends on contaminants solubility, molecular mass, concentration, polarity, pH, temperature, soil humidity, etc. [2, 3].

Nowadays there are experimental data obviously demonstrating plants potential to activate a definite set of biochemical and physiological processes to resist the toxic action of contaminants by the following mechanisms:

– Excretion

– Conjugation of contaminants with intracellular compounds and further compartmentalization of conjugates into cellular structures

– Decomposition of environmental contaminants to standard cell metabolites or their mineralization.

Commonly, plants gradually degrade entering cells organic contaminants to avoid their toxic action. According to contaminants assimilating potential plants are differing up to four orders of magnitude that allowed to classifying plants as strong, average and weak absorbers of different structure contaminants. For instance the most active assimilators uptake up to 10 mg of benzene per 1kg of fresh biomass per day, the assimilation potential of the weak absorbers is measured in hundredths of mg [4].

The fate of entered plant cell contaminants depends on their chemical nature, external temperature, variety of plants and phase of vegetation, etc. The simplest pathway of entered the plant cell organic contaminants is excretion. The essence of excretion is that the toxicant molecule does not undergo chemical transformation, and being translocated through the apoplast, is excreted from the plant. This pathway of xenobiotic (contaminant) elimination is rather rare and takes place at high concentrations of highly mobile (phloem-mobile or ambi-mobile) xenobiotics.

In the great majority, contaminants being absorbed and penetrated into plant cell undergo enzymatic transformations leading to the increase of their hydrophilisity-process simultaneously accompanied by decreasing of toxicity. Below are presented successive phases of contaminants initial transformations in accordance to Sandermann's green liver concept [5] (Fig. 1).

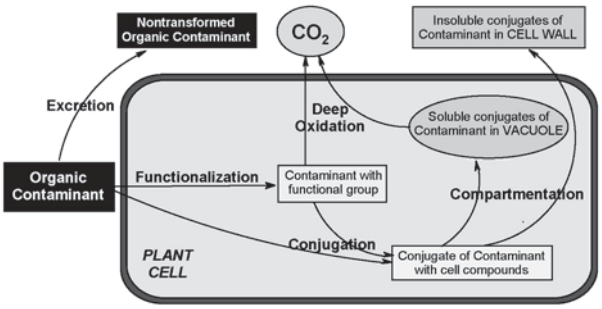


Fig. 1. **The main pathways of organic contaminant transformation in plant cells**

Functionalization is a process whereby a molecule of a hydrophobic organic xenobiotic acquires hydrophilic functional group (hydroxyl, carboxyl, amino, etc.) as a result of enzymatic oxidation, reduction, hydrolysis, etc. Due to the introduction of functional group the polarity and correspondingly reactivity of the toxicant molecule is enhanced. This promotes an increase of intermediates affinity to enzymes, catalysing further transformation.

Conjugation takes place a basic process in phytoremediation and consists in formation of chemically coupled contaminant to endogenous cell compounds (proteins, peptides, amino acids, organic acids, mono-, oligo-, polysaccharides, lignin, etc.) forming of peptide, ether, ester, thioether or other type covalent bonds. Intermediates of contaminants initial

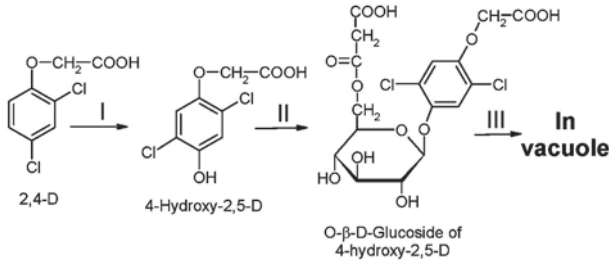
transformations or contaminants themselves possessing functional groups capable of reacting with intracellular endogenous compounds are susceptible to conjugation.

Commonly, immediately after penetration, the main part of the toxicant molecules undergoes conjugation and only a small amount is deeply degraded (0.1–5% depending on contaminants structure). Conjugation is a wide spread defence mechanism in higher plants especially in cases when penetrated into plant cell concentration of the contaminants exceeds the plant's transformation (decomposition) potential. Increased amount of deep degradation to regular plant sell metabolites, or CO_2 and water is achieved in case of linear, low molecular structures of contaminants [2]. The toxicity of conjugates compared to parent compounds is decreased due to binding with non-toxic cellular compounds. Conjugates are kept in a cell for a certain period of time without causing visible pathological deviations in cell homeostasis. Conjugates formation also gives the plant cell extra time for the internal mobilization, induction of enzymes responsible for contaminants further transformation. Relatively quickly, after the termination of plant incubation with the contaminant, conjugates are no longer found in plant cells.

Some attempts have been made by authors (unpublished data) to estimate different plant (soybean, ryegrass) cells potential to accumulate conjugated benzene in their cells in case of toxicant saturation. In spite of incomplete information it was suggested that for genetically non modified plants it could be, as a minimum, several molecules of contaminant conjugates per each plant sell. Although conjugation is one of the most widely distributed pathways of plant self-defence, it cannot be assumed as energetically and physiologically advantageous for the plant process. Firstly formation of conjugates leads to the depletion of vitally important cellular compounds, and secondly unlike deep degradation, formation of conjugates is maintaining contaminants basic molecular structure, and hence results only in partial and provisional decreasing of its toxicity.

Compartmentation in most cases the final step of conjugates processing temporary (short or long) storage of conjugates in defined compartments of the plant cell takes place. Soluble conjugates of toxic compounds (coupled with peptides, sugars, amino acids etc.) are accumulated in cell structures (primarily in vacuoles), while insoluble conjugates (coupled with, lignin, starch, pectin, cellulose, xylan) are moved out of the cell via exocytose in the apoplast being accumulated in cell wall [5]. The compartmentalization process is analogous to mammalian excretion, essentially removing toxic part from metabolic tissues. The major difference between detoxification in mammals and plants is that plants do not have a special excretion system for the removal of contaminants conjugates from the organism. Hence they use a mechanism of active transport for the removal of the toxic residues away from the vitally important sites of the cell (nuclei, mitochondria, plastids, etc.). This active transport is facilitated and controlled by the ATP-dependent glutathione pump [6] and is known as «storage excretion» [7].

The described above pathway of toxic compound processing i.e., functionalization → conjugation → compartmentalization, is well illustrated by the processing of anthropogenic contaminants of different structures. One of such examples demonstrating the transformation of organochlorine pesticides is the hydroxylation of 2,4-D followed by conjugation with glucose and malonyl residues and deposition in vacuoles [8].



2,4-D transformation for deposition in vacuoles

The Enzymes. Anthropogenic organic toxicants decomposition processes are closely related to many aspects of higher plants cellular metabolism. In prolonged and multifunctional detoxification processes quite a few enzymes are actively involved. According to catalyzed reactions they are directly or indirectly participating in detoxification process.

Ttransformations of contaminants during functionalization, conjugation and compartmentation are of enzymes function. It is remarkable that due to their unusual flexibility in the absence of xenobiotics, in plant cell these enzymes catalyse reactions typical for regular plant cell metabolism. Below are presented enzymes directly participating in the transformation process of anthropogenic contaminants:

– Oxidases, catalyzing hydroxylation, dehydrogenation, demethylation and other oxidative reactions (cytochrome P450-containing monooxygenases, peroxidases, phenoloxidases, ascorbatoxidase, catalase, etc.).

– Reductases, catalyzing the reduction of nitro groups (nitroreductase).

– Dehalogenases, splitting atoms of halogens from halogenated and polyhalogenated xenobiotics.

– Esterases, hydrolyzing ester bonds in pesticides and other organic contaminants.

Conjugation reactions of contaminants in plant cell are catalyzed by transferases: Glutathione S-transferase (GST), glucuronozyl-O-transferase, malonyl-O-transferase, glucosyl-O-transferase, etc. Compartmentation of intermediates of contaminants transformation-conjugates takes place under the action of ATP-binding cassette (ABC) transporters [9]. Depending on the structure of the contaminant some other enzymes may also be involved in their degradation process.

Prolonged in time cellular decomposition of contaminants involves participation of enzymes providing plant cell with extra energy needed for the defence processes, induction of the enzymes, and provision of cells by vitally important secondary metabolites. Enzymes involved in these and similar processes obviously indirectly participate in the detoxification of contaminants. The correlation between the penetration of organic contaminants (alkenes, aromatic hydrocarbons, polycyclic aromatic hydrocarbons) in plant cells and the corresponding changes in the activities of enzymes participating in energy supply (malate dehydrogenase) and nitrogen metabolism (glutamate dehydrogenase, glutamine synthetase) has been revealed. As it has been shown the activities of the enzymes are highly affected by xenobiotics concentration, exposure time and mode of illumination [10].

Ecologically the most advantageous pathway of organic contaminants transformation in plants is their deep oxidative degradation. In higher plants mainly the following enzymes are responsible for this process: cytochrome P450-containing monooxygenese, peroxidase and phenoloxidase. To correctly evaluate the universality of the action of these enzymes, responsible for the degradation of different structure organic contaminants, some of their specificities should be emphasized.

Cytochrome P450-containing monooxygenases (EC 1.14.14.1) are mixed-function enzymes located in the membranes of the endoplasmic reticulum (microsomes) [11]. Monooxygenase system contains redox-chain for electron free transport, the initial stage of electron transfer is a NADPH-cytochrome P450 reductase (EC 1.6.2.4); the intermediate carrier — cytochrome b_5, and the terminal acceptor of electrons — cytochrome P450. When NADPH is used as the only source of reductive equivalents, the existence of an additional carrier, a NADH-dependent flavoprotein is required. NADH may also be oxidized

Plants oxidative metalloenzymes

Enzyme	Physiological function	Existence in cell	Localization	Specificity to toxicants	Limiting factors	Stability
Cytochrome P_{450} containing monooxygenase	Participation in a number of intracellular synthesizing reactions	Small amount, inductive nature	Endoplasmatic reticulum, cytosole	Very high affinity to nonpolar toxicants	NADPH, NADH	Labile, inactivating during substrate oxidation
Peroxidase	Hormonal regulation, lignification, response on stress, removing of peroxides	Large amount, inductive nature	Cell wall, vacoules, cytosole, tonoplasts, plastids, plasmalemma	Affinity to aliphatic compounds	Hydrogen peroxide or organic hydroperoxides	Stable
Phenoloxidase	Oxidative transformation of phenols, lignification, cell defence reactions	Large amount, presents in latent form too, inductive nature	Chloroplasts, cell wall, cytosole, tonoplasts	Affinity to aromatic compounds	Endogenous phenols	Stable

by the NADPH-dependent redox system. In the latter case cytochrome b_5 is not required. The cytochrome P450-containing monooxygenases use NADPH and/or NADH reductive equivalents for the activation of molecular oxygen and incorporation of one of its atom into lipophilic organic compounds (XH) that results in formation of hydroxylated products (XOH) [12]. The second atom of oxygen is used for the formation of a water molecule (Fig. 2).

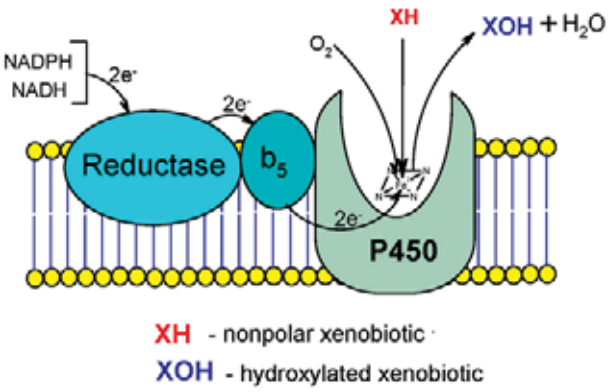


Fig. 2. **Microsomal monooxygenase system**

Plant cytochrome P450-containing monooxygenases play an important role in the hydroxylation of organic contaminants [5]. The enzymes participate in the reactions of C- and N-hydroxylation of aliphatic and aromatic compounds, N-, O-, and S-dealkylation, sulpho-oxidation, deamination, N-oxidation, oxidative and reductive dehalogenation, etc. [12, 13]. The resistance of plants against herbicides is mediated by their rapid intracellular transformation into hydroxylated products and subsequently conjugated to carbohydrate moieties in the plant cell wall. For examples, N-demethylation and ring-methyl hydroxylation of the phenylurea herbicide chlorotoluron in wheat and maize is cytochrome P450-dependent processes [14, 15]. For some phenylurea herbicides in the Jerusalem artichoke cytochrome P450-mediated N-demethylation is sufficient to cause significant or complete loss of phytotoxicity [16].

Peroxidase. In higher plants, peroxidase activity increases in response to stress. Among multiple functions of this enzyme one of major is the protection of cells from oxidative reactions imposed of all photosynthetic plants. The great catalytic versatility of the peroxidase is its predominant characteristic, and, therefore, no single role exists for this multifunctional enzyme.

The peroxidase is defined by the following reaction:

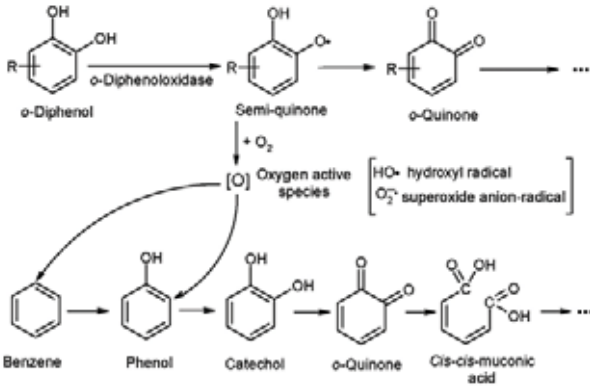
$$RH_2 + H_2O_2 \rightarrow 2H_2O + R.$$

The peroxidases catalyze a number of free radical reactions. Alternatively, the compound that is directly oxidized by the enzyme further oxidizes other organic compounds, including xenobiotics. This notion is based on the wide ubiquitous distribution of this enzyme in plants (the isozymes of peroxidase in green plants occur in the cell walls, plasmalemma, tonoplasts, intracellular membranes of endoplasmic reticulum, plastids and cytoplasm), and the high affinity and wide substrate specificity of plants peroxidases to organic xenobiotics of different chemical structures. In literature the participation of plant peroxidases in hydroxylation reactions of xenobiotics has been widely discussed. For example, peroxidases from different plants are capable of oxidizing N,N-dimethylaniline [17], 3,4-benzpyrene, 4-nitro-*o*-phenylendiamine [18], 4-chloroaniline [19], phenol, aminoflourene, acetaminophen, diethylstilbestrol, butylated hydroxytoluene, hydroxyanisoles, benzidine, etc. [5]; horseradish (*Armoracia rusticana*) peroxidase oxidizes tritium-labelled [C^3H_3] TNT [20].

Phenoloxidases, group of the copper-containing enzymes (other names-tyrosinase, monophenol monooxygenase, phenolase, monophenol oxidase, etc.) are spread within the plant cell organelles catalyzing both monooxygenase and oxygenase reactions: the *o*-hydroxylation of monophenols (monophenolase reaction) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase reaction). Currently accepted enzyme nomenclature classifies hydroxylating phenol oxidase as monophenol monoxygenase (EC. 1.14.18.1) and *o*-diphenols oxidizing phenol oxidase as catechol oxidase (EC 1.10.3.1). Plant phenol oxidases appear to be a group of specific enzymes, oxidizing wide range of *o*-diphenols, such as DOPA (dihydroxyphenylalanine), catechol, etc, but unable to convert *m*- or *p*- diphenols to the corresponding quinons, Substrate specificity of catechol oxidase from *Lucopus europaeus* and characterization of the bioproducts of enzymatic caffeic acid oxidation, FEBS Letters, 445, 103–110). The active center of phenol oxidases contains two cooper atoms and exists in three states: «met», «deoxy» and «oxy».

Phenoloxidases actively participate in the oxidation of xenobiotics of aromatic struc-

ture. As it has been demonstrated phenoloxidase from spinach, analogously to many other plants, oxidizes aromatic xenobiotics (benzene, toluene), by their hydroxylation and further oxidation to quinone [4]. In a number of the cases, if the xenobiotic is not a substrate for the phenoloxidase, it may undergo co-oxidation in the following manner: the enzyme oxidizes the corresponding endogenous phenol by forming quinones or semi-quinones or both, i.e. compounds with a high redox potential. These compounds activate molecular oxygen by forming oxygen radicals, such as superoxide anion radical (O^{2-}) and hydroxyl radical (·OH) [21], that gives compounds the capacity for the further oxidation of xenobiotic. The formation of these radicals enables phenoloxidase to participate in contaminants degradation processes also by co-oxidation mechanism presented below (Fig. 3).



Enzymatic oxidation of *o*-diphenols (upper) by phenoloxidase and non-enzymatic co-oxidation of benzene (lower)

Analogously, nitrobenzene is oxidized to *m*-nitrophenol, and the methyl group of [C_3H_3] TNT [20] is oxidized by phenoloxidase from tea plant. The information confirming participation of this enzyme in the oxidative degradation of xenobiotics in higher plants is sparse [4], despite the fact that participation of phenoloxidase should definitely be expected while xenobiotics degradation. Laccase of basidial fungi, analogous to higher plant phenoloxidase, have been better explored. Laccase degrades different aliphatic and aromatic hydrocarbons [22], and actively participates in the enzymatic oxidation of alkenes [23]. Crude preparations of laccase isolated from the white rot fungus *Trametes versicolor* oxidizes 3,4-benzopyrene, anthracene, chrysene, phenanthrene, acenaphthene and some other PAHs [24]. The intensity of oxidation of these antropogenic contaminants is increased in the presence of such mediators as: phenol, aniline, 4-hydroxybenzoic acid, 4-hydroxybenzyl alcohol, methionine, cysteine, reduced glutathione, and others compounds-substrates of laccase [25]. These data indicate that in the cases of fungal laccase and plant o-diphenoloxidase, the oxidation of hydrocarbons is carried out by a co-oxidation mechanism [4].

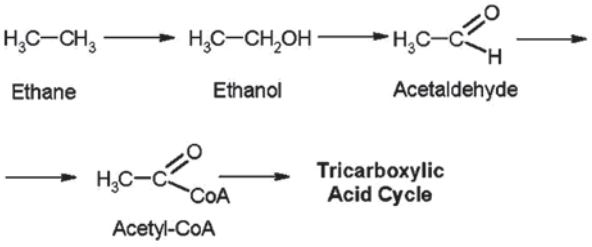
Apparently metalo-enzymes differing in their localization in plant cell organelles, structural organization, mechanisms of action, substrate specificity allow plants firstly to oxidise wide spectrum of organic contaminants including aromatic structures containing contaminants and secondly regulate inter-replacement of these enzymes during contaminants (xenobiotics) oxidative degradation caused due to inability or decreased potential of any of these enzymes to carry out further oxidation of structurally unsuitable intermediates.

Deep degradation of organic xenobiotics (contaminants) is multistage, mainly oxidative enzymatic process and only insignificant amount of toxic molecules undergo direct degradation, the majority of the conjugated with endogenous metabolites contaminants (above 80%) are accumulated in vacuoles and apoplasts and their further transformation takes place with some delay. The emission of $^{14}CO_2$ (up to 5% in case of labelled linear contaminants) indicates that in plant cells the formation of conjugates and their compartmentalization is followed by deep oxidation of the toxic parts of their molecules [4, 26].

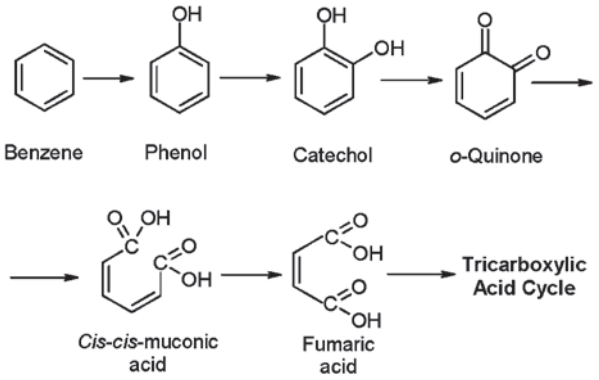
Based on the number of experimental data it is supposed that the most rate-limiting stage of the whole process of xenobiotics transformation seems to be the initial hydroxylation of nonpolar contaminants. As a result of functional group introduction molecule of transformed contaminants becomes easily accessible for further enzymatic transfomation.

The transformation of the small molecular weight aliphatic xenobiotics as methane in tea plant (*Thea sinensis*) proceeds by the formation of fumaric acid. Transformation of ethane, propane and pentane leads to the formation of low molecular mass compounds largely composed by di- and tricarbon organic acids. Labelled fumaric, succinic, malonic, citric and lactic acids are identified in plant leaves exposed to these low molecular mass alkanes, with most of the radioactivity incorporated into succinic and fumaric acids. The absence of oxalic acid directly indicates that ethane in plants is oxidized monoterminally.

The oxidation of ethane at one terminal carbon atom leads to the formation of acetyl-CoA, which in turn participates in the Krebs cycle [27].



Transformation of ethane in higher plants



Oxidative degradation of benzene in plant cells

Long chain alkanes are subjected to similar transformations. For instance, after 40 min of incubation of leek leaves with an emulsion of exogenous [^{14}C] octadecane in water, 9.6% of the total label is detected in esters, 6.4% in alcohols, and 4% in organic acids [28].

The most significant input in understanding in plants detoxification process has been revealed by discovery of plants ability to transform (oxidatively decompose) benzene and phenol via aromatic ring cleavage. As a result of such degradation carbon atoms of contaminants are incorporated into organic acids and amino acids. Similar data were reported for nitrobenzene, aniline, toluene, α-naphthol, and benzidine transformation in plants [29, 30]. Oxidation of benzene and phenol by crude enzyme extracts of plants forms muconic acid as a result of ring cleavage, with catechol formation, as intermediate.

Further oxidation of muconic acid results in formation of fumaric acid. Labelled muconic and fumaric acids are found in plants exposed to labelled benzene or phenol. Cleavage of the aromatic ring in endogenous substrates proceeds by the transformation of 3,4-dihydroxybenzoic acid into 3-carboxymuconic acid [31]. Phenoxyalkyl-carboxyl acids containing four and more carbon atoms in their side chain often undergo β-oxidation in plants. For instance, 2,4-dichlorophenoxybutyric acid is oxidized resulting by formation of 2,4-D [32–34].

Finally contaminants degradation proceeds to standard cell metabolites or mineralization. Degrading xenobitic the plant cell not only avoids its toxic action but also utilizes its carbon, nitrogen, and other atoms for intracellular biosynthetic and energetic needs. The totality of such transformations is the essence of the plants detoxification process. Direct complete xenobiotic degradation in a plant cell is however accomplished only at low, metabolic, concentrations of environmental contaminants and respective time (it may last days or weeks).

Ultrastructure. To evaluate the ecological potential of plants, the data proving the responses at the level of cell ultrastructure under the action of contaminants, as the most precise indications of plants exploitation, should also be emphasized. Undoubtedly, penetration even a small concentrations of contaminants into plant cells leads to invisible, but most often measurable deviations in cell metabolic processes such as: induction of enzymes, inhibition of some intracellular metabolic processes, change the level in regular secondary metabolites, etc. The existence in plant cell contaminants in increased concentrations provokes clearly noticeable deviations in cells ultrastructural organization. It has been shown that the complex of changes and alterations in the main metabolic processes of plant cell elicited by organic pollutants (pesticides, hydrocarbons, phenols, aromatic amines, etc.) are connected with the deviations of cell ultrastructural architecture. The sequence and deepness of the destruction in plant cell organelles are determined by the variety of plant, chemical nature, concentration and duration of the contaminant action, etc. [35, 36]. This course of events has been experimentally demonstrated in a number of various higher plants exposed to different ^{14}C-labelled toxic compounds. In these experiments due to the penetration, movement and localization of contaminants in plant cells changes in ultrastructural organization has been shown. Apparently, the negative affects of toxic compounds on cell ultrastructure, depending on its concentration, could be divided on two

types, being different for each contaminant and plant:

– metabolic, which is digested by the plant in spite of insignificant negative effect by the mobilization of plants internal potential

– lethal, leading to indigestible deviations and to the plant death.

On the Fig. 3 is shown maize root apex cells exposed to ^{14}C-nitrobenzene action, its penetration across the plasmalemma and localization in subcellular organelles. Studies of penetration of ^{14}C–labelled xenobiotics into the plant cell indicate that labelled compounds at the early stages of exposure (5–10 min) are detected in the cell membrane, in the nuclei and nucleolus (in small amounts), and, seldom, in the cytoplasm and mitochondria. As a result of prolonged exposure the amount of a label significantly increases in the nucleus, at the membranes of organelles, in tonoplasts, and further in vacuoles, i.e. xenobiotic becomes distributed in most of subcellular organelles, but ultimately there is a tendency of contaminants primary accumulation in vacuoles.

The general picture of the evolving action of organic contaminants on plant cells with duration of exposure is the following:

Initially, changes in the configuration of the nucleus become noticeable. Simultaneously inhibition of DNA synthesis takes place. The barrier function of the plasmalemma and its ability to accumulate calcium are damaged. Ca^{2+} concentration in the cytoplasm is increased; Ca^{2+}–ATPase activity is inhibited. Mitochondria with swollen cristae and packed matrix becomes noticable, the plastids are electron-dense and enlarged.

Prolonged action of contaminants leads to a widening of the cisternae of the endoplasmic reticulum and Golgi apparatus, vacuolization of the cytoplasm. The size of cytoplasm is thereby decreased and the periplasmic space concomitantly enlarged. In some cortical cells of the root apices, the number of ribosomes in the hyaloplasm is increased, and the formation of polysomes is observed. Lysis of mitochondria and depletion of ribosomes from the endoplasmic reticulum of membranes take place. Multiple contacts between the endoplasmic reticulum and the plasmalemma, vacuoles, nucleus, and membranes of the mitochondria are detectable. The enhancement of the size of the nucleus and chromatin coagulation, indicating a disturbance of the DNA synthesis process, is observed. Nuclei acquire deviant shapes because of the development of many protuberances of the nuclear membrane. In leaf cells, chloroplast shape and composition

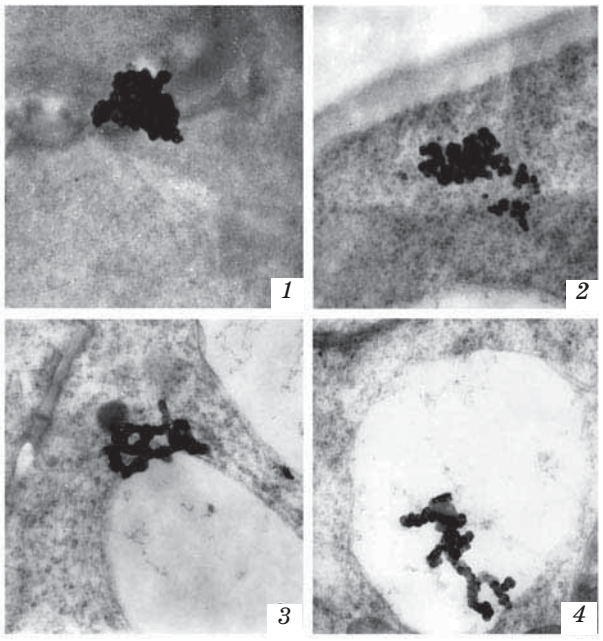


Fig. 3. **Electron micrographs showing the penetration and movement of ^{14}C-labelled nitrobenzene (0.15 mM) in a maize root apex cell**
The xenobiotic penetrated through the plasmalemma (*1*), moved to the cytoplasm (*2*), and thereafter translocated into vacuoles (*3, 4*).
1 — ×48 000; *2* — ×36 000;
3 — ×50 000; *4* — ×30 000

become ill defined, the external membrane is not visible, the orientation of the system is disturbed, and matrix is brightened with large osmiophilic inclusions. In the cytoplasm accumulation of the differentiated cells of the root caps that secrete mucus, is visible. Some of these hypertrophied vesicles are fused forming a large deposit of mucus. Inhibition of the process of maturing secretory vesicles translocation towards the cell periphery is often correlated not only with the swelling of vesicles, but also with the disappearance of the normal dictyosomes.

Prolonged exposure to environmental contaminants causes extensive destruction of the cell and plant death.

Obviously plants, as remediators, for a long time the most effectively act at low and shallow contamination of soil and air, when no significant changes in cell ultrastructure might be detected. Nevertheless, it should be underlined that plants subjected to high concentrations for relatively short periods in most cases are able to recover from slight deviations in cell ultrastructure and thus maintain their vital activities.

Phytoremediation is a unique cleanup strategy. The realization of phytoremediation

technologies implies the planting on a contaminated area with one or more specific, previously selected plant species with the potential to extract contaminants from soil. A precise survey of the vegetation *on site* should be undertaken to determine what species of plants would have the best growth at the contaminated site. Based on the number of experimental results including the use of labeled xenobiotics and electron microscopic observations, the deep degradation of anthropogenic contaminants in higher plants could be considered as narrow but permanently working pathway having much less potential than conjugates formation process (especially in case of contaminants saturation).

During the last decade phytoremediation from a conceptual methodology has become into ecologically important commercial technology for the cleaning of environment. The successful realization of phytoremediation technologies greatly depends on the synergetic action of microorganisms and plants. In order to increase the ecological potential of plants, definite progress has already been achieved by the cloning of genes of the enzymes participating in contaminants transformation/accumulation. A number of genetically modified plants having especially high accumulation potential and correspondingly large intracellular volume to deposit metabolite — xenobiotic conjugates have been created. Some publications [37, 38] are devoted to the discussion of these and other problems concerning the uptake of inorganic contaminants. In these publications where transgenic plants, characterized by enhanced tolerance to cadmium and lead (70–75 mM), which inevitably points to their hyperaccumulation potential, are described. Data indicate the doubling of the lead content in transgenic plants has also been detected [39].

Among the large diversity of plants with perspectives for phytoremediation the poplar family attracts special interest. Owing to its strong root system it is characterized by the increased absorption ability. Multiple gene-engineering modifications of this plant have presented convincing evidence for the expediency in practical usage of some plants-transformants generated. Cloning of Glutathione S-transferase was successful in creation of several perspective transgenic clones. The transfer of cytochrome P450 genes to different plants has been a wide spread activity for last decade [40]. Some of the created transgenic plants generally are characterized by high resistance to herbicides of different structure and have clearly observable high detoxification potentials [13].

Transgenic plants have also been studied in connection with degradation of several (some) particular contaminants. For this purpose the widely distributed explosive TNT has generally been chosen. In order to increase the degradability of TNT and similar compounds, the transgenic plants (several) contained the gene of bacterial enzyme (pentaeritrole tetranitrate reductase, EC 1.6.99.7) were received [41]. Transgenic tobacco has been analysed for its ability to assimilate the residues of TNT and trinitroglycerine. Seedlings of the transgenic plants extracted explosives from the liquid area much faster, accomplishing denitration of nitro groups, than the seedlings of common forms of the same plants, in which growth was inhibited by the contaminants [42]. Transgenic tobacco thus differed substantially from the common plant by its tolerance, fast uptake and assimilation of significant amounts of TNT. Analogous experimental results have been obtained with other plant species [43].

There are douzens of publications concerning successful improvement of plant detoxification abilities by cloning the genes of transferases and oxidases, which intensively participate in contaminant transformation processes [13, 40].

Obviously, attempts to improve artificially ecological potential of higher plants will be continued, and the results will be the more substantial from the viewpoint of their eventual practical realization. The positive effect of these investigations could be much more impressive if all aspects of the quite complicated and multistage detoxification process would be better elucidated with regard to plants physiology and biochemistry. Such information would allow the creation of more rational and effective strategy for the gene engineering technique application.

Until recently plants were considered as organisms having a naturally limited potential for contaminants conjugation and accumulation. Last decade have clearly revealed the potential of plants to absorb and decompose organic contaminants and accumulate inorganic contaminants from soil, water and air. Depending on the nature of the organic xenobiotic and the type of plant, typically 1 kg of green biomass takes up from the air daily amounts ranging from microgram's to tents of milligrams of pollutants [2, 4, 44]. Plants possessing the universal cleaning up (i.e. applicable to soil, groundwater and air) capabilities are the only agents carrying out the process of

remediation by transporting metals to above ground parts of plants. Some plants are indeed known as hyper accumulators of metals. For the superterranean instance transgenic plants of Indian mustard, poplar, tobacco, *Thlaspi*, *Arabidopsis*, etc. possess especially high potentials for metal accumulation and transportation [39, 45, 46].

Elimination of contaminants located deeper than two metres in the soil is connected with limitations in time, since mass transfer processes at that depth and deeper proceeds much more slowly than in upper parts. Hence extraction by roots and the subsequent transport may become the rate-limiting factor of the whole process. Therefore, plant-microbial action-based technologies would need excessive time to achieve a satisfactory clean standard of soil. In case of contaminants high concentration, phytoremediation as a final «polishing step» must follow other technologies such as excavation, treatment or disposal, etc. Other case when phytoremediation is not successfully applied is the high concentrations of soil contaminants such as polychlorinated biphenyls and dioxins. At high concentrations of these compounds no plants can grow up in contaminated soil. In such extraordinary cases phytoremediation technology alone in any realistic time cannot clean up the soil.

On the other hand plants are very promising detoxifiers *qua* ecologically safe technologies around hotbeds of contamination [2], Vegetation cap, Phytoremediation cover, Hydrologic control, Evapotranspiration cover or any other plant based technology) — ecologically friendly and of significant ecological importance. Elaboration of a new ecological concept, unifying worldwide experience accumulated for last 30 years and realizing of new plant-based approaches in the world scale should lead to the increase of the ecological potential of the whole planet. The universality of phytoremediation consists in the uptake nearly of all types of organic contaminants and heavy metals and their accumulation in intracellular structures or oxidative mineralization.

Owing to the still wide terrestrial and aquatic distribution of plants we should consider these organisms as a very important biological instrument having tremendous ecological potential.

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ДЕГРАДУВАННЯ АНТРОПОГЕННИХ КОНТАМІНАНТІВ ВИЩИМИ РОСЛИНАМИ

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Еволюційно зумовлена здатність мікроорганізмів різних таксономічних груп до елімінації забруднювальних речовин з навколишнього середовища загальновідома й широко обговорюється в літературі. Водночас донедавна вважали, що рослини, які займають близько 40% суші, мають обмежений потенціал зв'язування забруднювальних речовин і накопичення їх усередині клітинних органел. Автор статті, ґрунтуючись на 40-річному досвіді роботи в цій галузі, розглядає з погляду сучасних уявлень різні аспекти екологічного потенціалу рослин; механізм заміни ензимів, що беруть участь в оксидативній деградації органічних забруднювальних речовин; роль у цьому процесі фенолоксидази; критерії оцінки потенціалу рослин до детоксифікації за таким точним індикатором, як зміна рослинних клітин на ультраструктурному рівні під дією забруднювальних речовин.

Ключові слова: вищі рослини, антропогенні забруднювальні речовини, оксидативна деградація, фенолоксидаза.

ДЕГРАДАЦИЯ АНТРОПОГЕННЫХ КОНТАМИНАНТОВ ВЫСШИМИ РАСТЕНИЯМИ

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Эволюционно обусловленная способность микроорганизмов различных таксономических групп к элиминации загрязняющих веществ из окружающей среды общеизвестна и широко обсуждается в литературе. В то же время до недавнего времени считали, что растения, занимающие около 40% суши, обладают ограниченным потенциалом для связывания загрязняющих веществ и накопления их внутри клеточных органелл. В представленной статье автор, основываясь на 40-летнем опыте работы в данной области, рассматривает с точки зрения современных представлений различные аспекты экологического потенциала растений; механизм замены энзимов, участвующих в оксидативной деградации органических загрязняющих веществ; роль в этом процессе фенолоксидазы; критерии оценки потенциала растений к детоксификации по такому точному индикатору как изменение растительных клеток на ультраструктурном уровне под действием загрязняющих веществ.

Ключевые слова: высшие растения, антропогенные загрязняющие вещества, оксидативная деградация, фенолоксидаза.

UDK 577.083.3+579.84

POLYADHESINS: AN ARMORY OF GRAM-NEGATIVE PATHOGENS FOR PENETRATION THROUGH THE IMMUNE SHIELD

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The rapid emergence of treatment-resistant bacterial pathogens has become a major threat to public health. The outbreak of new Shiga-toxin–producing *Escherichia coli* O104H4 infection occured in Germany in 2011 illustrates this problem. To colonize host tissues, pathogenic bacteria express surface adhesive organelles. The German strain uses aggregative adherence fimbriae I (AAF/I) to anchor to the intestinal mucosa and induce inflammation. AAF/I belong to the family of chaperone/usher assembled fimbrial polyadhesins. Polyadhesins are functioning as an armory for penetration through the host immune shield. The polyadhesin-binding to the target cells triggers subversive signal by aggregation of host cell receptors that allow pathogens to mislead and evade immune defense. Their binding is orchestrated with the type III secretion system, which is extremally important for bacterial virulency. Polyadhesins also are involved in biofilm formation making bacteria more resistant to immune response. Because of this, the polyadhesins are potential targets for immune countermeasures against bacterial infections, in particular for anti-adhesion therapy with antibodies to polyadhesins as one of alternatives to antibiotic therapy.

Key words: Gram-negative pathogens; polyadhesins; anti-immune armory.

Adhesive organelles of bacterial pathogens are crucial virulence factors, mediating attachment to the target cells of their hosts and initiating infectious process. They also are involved in biofilm formation making bacteria more resistant to immune response.

Gram-negative pathogens possess two major classes of proteinaceous adhesins [1]:

- The fimbrial adhesive organelles, represented by the linear homopolymers or heteropolymers (up to 7 distinct subunits) of hundreds to thousands of protein subunits;
- The non-fimbrial adhesins consisted of a single protein or homotrimers.

The superfamily of fimbrial organelles, assembled by the chaperone/usher (CU) machinery, is divided in two functionally distinct families: monoadhesins and polyadhesins [1, 2].

Monoadhesins comprises in main the thick rigid and thin flexible adhesive pili of a complex subunit composition (up to 7 distinct subunits), which typically display only one adhesive domain on the tip of the pilus. The assembly of monoadhesins is assisted with the FGS (having a short F1-G1 loop) class of periplasmic chaperones [3, 4]. The monoadhesins are encoded in main by the gene clusters of the γ1-, γ2-, γ4-, and γ-monophyletic groups [5].

Polyadhesins, typically, have non-pilus, amorphous or capsule-like morphology. They either comprise homopolymers, which consist of only one type of subunit, or heteropolymers, which consist up to 6 distinct subunits. The notable property of polyadhesins is that all subunits of homopolymers or one of the main structural subunits of heteropolymers possesses one or two independent binding sites specific to different host cell receptors [1, 2]. Assembly of one subfamily of polyadhesins is assisted with the FGL (having a long F1-G1 loop) class of periplasmic chaperones [3, 4]. FGL chaperone-assembled polyadhesins are encoded exclusively by the gene cluster of the

γ3-monophyletic group [5]. The assembly of another subfamily of polyadhesins is assisted with the FGS class of chaperones. FGS chaperone-assembled polyadhesins are encoded in main by the gene cluster of the κ-monophyletic group [5]. Recently a novel member of polyadhesin family, the *Escherichia coli* common pilus (ECP), has been revealed [6]. The ECP has the unique architecture. It is composed of two sequentially combined polyadhesive homopolymers of EcpA and EcpD subunits, recpectively, with a dual role in biofilm formation and host cell recognition. The ECP is assembled via alternative CU pathway [7] and encoded by the gene cluster related to the α-monophyletic group [5].

Polyadhesins are functioning as an armory for penetration through the host immune shield. The polyadhesin-binding to the target cells triggers subversive signal by aggregation of host cell receptors that allow pathogens to mislead and evade immune defense [1, 2]. Their binding is orchestrated with the type III secretion system, which is extremally important for bacterial virulency [8]. Polyadhesins also are involved in biofilm formation making bacteria more resistant to immune response [6]. Because of this, the polyadhesive organelles are potential targets for immune countermeasures against bacterial infections, in particular for anti-adhesion therapy with antibodies as one of alternatives to antibiotic therapy [9, 10].

Several excellent reviews focused on the results of the structure/functional studies of FGS-chaperone assembled fimbrial monoadhesins have been published recently. Among the later were the reviews by [11–15]. However, the last comprehensive review on the structure, function, phylogenesis and clinical applications of polyadhesins was published by us more than three years ago [1]. The recently accumulated significant knowledge on different aspects of biogenesis of the growing family of Gram-negative polyadhesins and their clinical applications requires a new analysis and generalization.

ORGANIZATION OG GENE CLUSTERS ENCODING POLYADHESINS

Genes of proteins involved in the expression and assembly of polyadhesive fibers via the CU pathway are arranged into compact gene clusters, which are located either on the chromosome or on the plasmids of Gram-negative bacteria. Depending on the structural properties of periplasmic chaperones and phylogenetic classification, suggested by [5], they can be divided into three families:

- FGL chaperone-comprising gene clusters related to the γ3-monophyletic group;
- FGS chaperone-comprising gene clusters related to the κ-monophyletic group;
- Alternative chaperone-comprising gene cluster(s) related to the α-monophyletic group.

FGL Chaperone-Comprising Gene Clusters related to the γ3-Monophyletic Group

Our studies, which opened the way to finding a family of polyadhesins, began with the cloning and sequencing of the genes responsible for the formation of the capsule of *Yersinia pestis*, the causative agent of pneumonic plague.

Encoded by the *caf* gene cluster fraction 1 (F1), capsular antigen from *Y. pestis* comprises aggregated high-molecular-weight linear polymers of a single subunit Caf1 [1, 2, 8, 16-18]. The genes of the *caf* gene cluster, *caf1*, *caf1M*, *caf1A* and *caf1R*, encode, respectively, for Caf1 subunit, periplasmic chaperone Caf1M, an outer membrane assembler, the molecular usher Caf1A and the protein Caf1R regulating gene cluster transcription [19–32].

The *psa* gene cluster from *Y. pestis* encodes proteins for expression and assembly of the fimbrial pH6 (Psa) antigen comprising the high-molecular-weight polymer of the PsaA subunit [33]. PsaB functions as the periplasmic chaperone and PsaC as the molecular usher. Two additional proteins, PsaE and PsaF, have been shown to regulate the transcription of the *psaA* gene [34]. Another transcriptional regulator, RovA, interacts with the *psaE* and *psaA* promoter regions, suggesting that RovA is an upstream regulator of the *psa* gene cluster [35]. Identical *psa* gene clusters are present in *Y. pestis* and *Y. pseudotuberculosis* [33].

Closely related to the *psa* gene cluster of *Y. pestis*, *Y. enterocolitica* contains *myf* encoding the Myf fimbriae, which are built up of MyfA subunits [36]. The *psa* and *myf* clusters have a similar general organization. Moreover, proteins encoded by these gene clusters display a significant sequence similarity, suggesting that the pH6 (Psa) antigen and Myf fimbriae have a common function in the different species of *Yersinia*. Like PsaE and PsaF encoded by *psa*, the MyfE and MyfF proteins encoded by *myf* play a role in the regulation of cluster transcription [37].

The *cs-3* gene cluster from *E. coli* encodes for proteins for expression and assembly of the

colonization factor-3 that forms CS-3 fimbriae comprising the high-molecular-weight polymer of the CS-3 subunit [38]. CS3-E functions as the periplasmic chaperone and CS3-D as the molecular usher.

The *nfa* gene cluster from *E. coli* encodes proteins for the expression and assembly of the nonfimbrial adhesin, NFA-I, comprising the high-molecular-weight polymer of the NfaA subunit [39]. NfaE functions as the periplasmic chaperone and NfaE as the molecular usher.

A group of *E. coli* gene clusters, *afa-3*, *afa-8*, *agg*, *aaf*, *agg-3*, *dafa*, *dra* and *daa*, which encode proteins for the expression and assembly of the afimbrial adhesins Afa-III and AfaE-VIII, the aggregative adherence fimbria type I, II and III (AAF/I, AAF/II and AAF-III), the diffuse adherence fibrillar adhesin (Dafa), the Dr hemagglutinin flexible fimbriae and the F1845 (DaaE) fimbrial adhesin, respectively, have a peculiar feature: each gene cluster encodes additional subunit D, for which an invasive function was suggested (putative invasin subunit) [40, 41]. DraE and AfaE-III adhesins may assemble into a flexible fiber, which provides the link between the usher at the outer membrane and the putative invasion subunit located at the tip of the fiber [42–44]. However, expression of DraD invasin subunit is independent of the DraC usher and DraE fimbrial subunit [45]. In addition, polymerization of DraE fimbrial subunits into fimbrial structures does not require the expression of DraD. Then, it was shown that type II secretion in *E. coli* strain Dr1 leads to DraD translocation to the bacterial cell surfaces [46]. Later, it was demonstrated that the DraD subunit is not required for β1 integrin recruitment or bacterial internalization [47, 48]. Therefore, the function of D subunits is still in question.

The *Salmonella* spp. gene clusters *saf*, *sef*, *cs6-1* and *cs6-2*, which encode proteins for the expression and assembly of the atypical fimbriae Saf, the filamentous fimbriae-like structures SEF14/18 and the colonization factors CS6-1 and -2, respectively, have another common peculiar feature: all of these gene clusters encode two adhesin subunits. The SefB chaperone of *S. enteritidis* assists in the assembly of two distinct cell-surface structures, SEF14 and SEF18, which are homopolymers of SefA and SefD subunits, respectively [49]. The CssC chaperone assists in assembling thin CS6 fibrillae, which are composed of two heterologous CssA and CssB subunits [50].

FGS Chaperone-Comprising Gene Clusters related to the κ-Monophyletic Group

The gene cluster *pef* is responsible for expression of plasmid encoded (PE) fimbriae of *S. typhimurium* composed of only one structural subunit, which probably functions as an adhesin subunit. A cosmid carrying the *pef* operon was introduced into *E. coli* and expression of fimbrial filaments composed of PefA was confirmed by flow cytometry and immune electron microscopy [51]. PE fimbriae were purified from the surface of *E. coli* and the resulting preparation was shown to contain PefA as the sole major protein component. Binding of purified PE fimbriae to a glycan array suggested that this adhesin specifically binds the trisaccharide Galss1–4 (Fuca1–3) GlcNAc, also known as the Lewis X (Lex) blood group antigen.

The gene clusters *fan*, *lda*, *fae* and *ral* encode proteins for the expression and assembly of the F4 (K88), Lda and F5 (K99) thin flexible pili and rabbit-specific enteropathogenic *E. coli* (REPEC) fimbriae of *E. coli*, respectively [52–54]. These pili/fimbriae consist of four or five subunits. However, F4 (K88), F5 (K99) and Lda pili do not display specialized adhesive domains on the tip of the pilus, but carry binding sites on their main structural subunit (FanG, FaeG and LdaG) [52, 54, 55]. The overall arrangement of the *ral* gene cluster closely resembles that of the *fae* cluster, with homologous genes occupying the same relative position in each cluster. The *ral* cluster also has some of the more specific features of the *fae* cluster, such as the overlapping reading frames of the genes encoded chaperone and usher and the apparent absence of promoters within the region carrying the structural genes [53]. This general similarity, together with the significant levels of homology exhibited by individual genes, makes it reasonable to propose functions for the *ral* gene products based on the known roles of their Fae counterparts. Thus, it was proposed that RalC, RalF and RalH are minor fimbrial subunits of the fimbrial structure, which is primarily composed of RalG, the major fimbrial subunit [53]. The gene cluster *afr* encodes proteins for the expression and assembly of the *E. coli* AF/R1 pili [56]. The subunits encoded by the *afr* gene cluster have the highest percentage amino acid identity with the subunits encoded by the *ral* cluster [53].

The *fed* gene cluster, encoding the F18 fimbriae, is composed of five genes, encoding the major subunit FedA, the usher protein FedB, the periplasmic chaperone FedC, the minor

pilin FedE and the adhesin FedF [57, 58]. Based on usher phylogeny, the *fed* cluster falls into the κ-fimbrial clade of CU systems [5]. However, it was demonstrated [59] that FedF, the F18 adhesin responsible for ABH glycosphingolipid binding, is a two domain adhesin typical for monoadhesive fimbrial organelles.

Alternative Chaperone-Comprising Gene Cluster(s) related to the α-Monophyletic Group

The most characterized member of this new family, *ecp* (or *mat*) gene cluster, encodes the *E. coli* common pilus (ECP), composed of two sequentially combined polyadhesive homopolymers of EcpA and EcpD subunits, respectively, with a dual role in biofilm formation and host cell recognition [6]. The *ecp* operon is composed of six genes: *ecpR, ecpA, ecpB, ecpC, ecpD*, and *ecpE*. Examination of EcpR revealed its function as transcriptional regulator [60], whereas primary sequence analysis of EcpB, EcpC, and EcpE [6] detected low but significant similarity with a variety of chaperone and usher proteins from the CU family [5]. The typical sequence identity is <17% with all known usher (EcpC) and chaperone (EcpB and EcpE) structures. Although sequence analysis of EcpD (~60 kDa) revealed no matches with other proteins, inspection of the N termini in EcpA and EcpD showed a significant level of similarity [6]. The *mat* (or *ecp*) gene cluster was related to the α-monophyletic group of alternative CU pathway [5, 7].

The ECP is highly conserved between enteric bacterial species [6]. *Serratia proteamaculans*, *S. odorifera*, *Klebsiella sp.*, *K. pneumoniae*, and *Enterobacter cancerogenus* possess fimbrial subunits with high homology to EcpA (80%, 82%, 98%, 97%, and 96% sequence identity, respectively), which may suggest a role for ECP in stabilizing multispecies biofilms by interactions through EcpA orthologs.

STRUCTURE OF POLYADHESIN SUBUNITS

Structure of Chaperone-Complemented Subunits

Chaperone-free subunits of fimbrial polyadhesins [18, 24] and monoadhesive fimbriae/pili [61, 62] are highly unstable and prone to form aggregates. Hence, structural information on many subunits of these organelles was obtained by studying chaperone–subunit complexes [17, 18, 63–67].

Figure 1 shows ribbon diagrams of the Caf1M–Caf1 [17, 18] and SafB–SafA [66] complexes. These complexes reveal the chaperone-bound conformation of FGL chaperone-assembled polyadhesin subunits. The polyadhesin subunits Caf1 and SafA have an incomplete immunoglobulin-like fold. Despite the lack of significant sequence similarity, polyadhesin subunits display similar organization of the B, C, E and F β-strands, which are known to form a common structural core of the immunoglobulin-like fold [68]. End of the F1–G1 loop and the beginning of the G1 β-strand in Caf1M harbor a subunit-binding motif of five alternating bulky hydrophobic residues (V126, V128, V130, F132 and I134) [17, 18]. The same region in the SafB molecule harbors a similar five-residue motif of one small hydrophobic (A114) and four bulky hydrophobic residues (L116, L118, L120 and I122) [66]. The rest of the F1–G1 loop (residues 104–123 in Caf1M and 104–113 in SafB) is disordered in the crystal structures. Another subunit-binding motif in FGL chaperones of three alternating hydrophobic residues (Y12 in Caf1M/F12 in SafB, V14 and I16) is localized in a long N-terminal sequence, which forms the A1 strand. A1 and G1 β-strands are the edge strands of the β-sandwich fold of the N-terminal domain. In the complex A1 and G1, β-strands are extended due to the partial

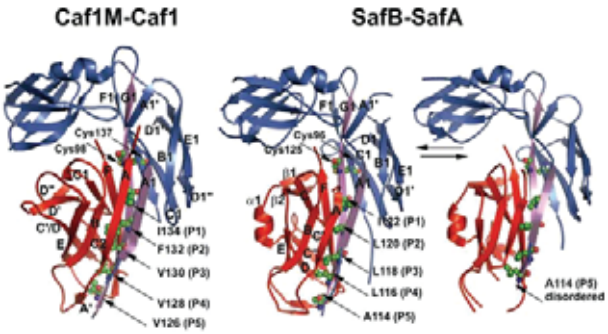

Fig. 1. **Ribbon presentation of the crystal structures of Caf1M–Caf1 and SafB–SafA complexes.** The chaperones are blue with G1 and A1 edge strands in violet; the subunits are red. The two conserved Cys residues in the whole FGL family that form disulfide bond are shown as ball-and-stick. The hydrophobic residues in the G1 strand of the chaperones that interact with the P5–P1 pockets of the subunits are also shown as ball-and-stick. The SafB residue A114, which interacts with the P5 pocket, is in equilibrium between a bound (left, type I structure) and an unbound (right, type II structure) state in the P5 pocket. The structures were redrawn based on the coordinates of atoms published by [17, 18] (PDB accession number 1P5V) and [66] (PDB accession numbers 2CO6 and 2CO7). All figures were prepared with PYMOL [69]

ordering of the N-terminal sequence and the F1–G1 loop, respectively, to form a binding platform, exposing the hydrophobic residues of the binding motifs. In addition to this binding structure, Caf1M and SafB chaperones apply a pair of conserved positively charged residues (R20 and K139 in Caf1M and R20 and K127 in SafB) to bind subunits by anchoring their C-terminal carboxyl groups.

Fig. 2, *A, C* illustrate how the Caf1 subunit is complemented by the Caf1M chaperone [17, 18]. The absence of the seventh (G) strand results in a six-stranded β-sandwich where the hydrophobic core of Caf1 is partially exposed in a long and deep hydrophobic groove. Caf1 interacts mainly with the N-terminal domain in Caf1M (Fig. 1). These two proteins bind via edge strands in Caf1 and in the N-terminal domain of Caf1M to form a closed barrel with a common core [17]. Strand G1 in Caf1M is hydrogen-bonded to strand F in Caf1. Chaperone A1 strand is hydrogen-bonded to subunit strand A. Hydrophobic residues from the Caf1M chaperone G1 strand are donated to the Caf1 subunit to compensate for the missing G strand (Fig. 1 and 2). The longer G1 donor strand of the Caf1M chaperone inserts a motif of five bulky hydrophobic residues (P1–P5 residues; Fig. 1 and 2) into five binding pockets in the hydrophobic groove of the Caf1 subunit (P1–P5 binding pockets).

The crystal structures of the Caf1M–Caf1 and SafB–SafA complexes show a considerably larger interactive area between the chaperone and the subunit than that found in the FGS chaperone–pilin complexes [63–65, 67]. This is a result of the presence of a more extended hydrophobic groove in the Caf1 and SafA subunits than in pilus subunits, which is complemented by subunit-binding motifs of Caf1M and SafB containing the additional FGL-specific sequences. However, the major F1–G1–loop–G1 β-strand-binding motif of SafB contains four rather than five bulky hydrophobic residues (L116, L118, L120 and I122), which interact with the hydrophobic P4–P1 pockets of the subunit's groove. The fifth donor residue inserting into pocket P5 is a small A114. Two crystal forms of the SafB–SafA complex were observed that differ in the extent of ordering around A114 (Fig. 1) [66]. In type I crystals, A114 is ordered and is inserted into the P5 pocket of the SafA subunit (Fig. 1). In type II crystals, this residue is disordered and does not insert into the P5 pocket (Fig. 1). As a result, the loops and secondary structure elements in the SafA subunit that form this P5 pocket are also disordered

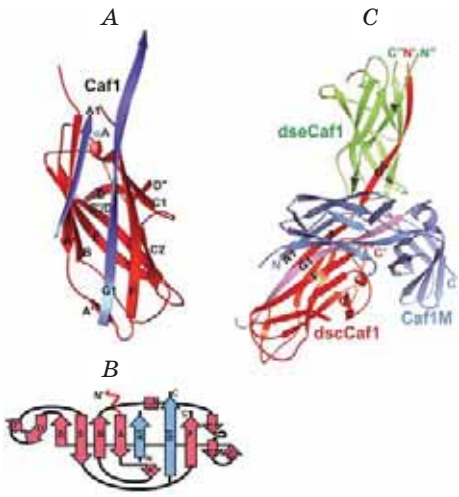

Fig. 2. **Structure of the Caf1 polyadhesin subunit complemented with Caf1M chaperone (*A, C*).** The Caf1 polyadhesin subunit is shown in red with donor strands from Caf1M chaperone (blue). Only interacting chaperone–subunit strands are shown. *B* — Ribbon diagram of the native Caf1M–Caf1′–Caf1″ complex. Caf1M is blue, except for G1 and A1 β-strands (violet). The chaperone-bound Caf1′ subunit and N-terminal donor strand (Gd) are red; the Caf1″ subunit corresponding to the tip of growing fiber is green. The N- and C-termini are labeled in the same colours as the ribbons. The redrawing is based on the coordinates of atoms of structures published by [17, 18] (PDB accession numbers 1P5V and 1Z9S). *A* and *C* were prepared with PYMOL [69]

and are not observed in the electron density map. These two structures suggest equilibrium between the two states of the SafB–SafA complex as a result of a weak binding of the chaperone G1 donor strand at the P5 site of the SafA-binding groove [66].

Structure of Subunits in the Fiber

The elucidation of crystal structure of *Y. pestis* F1 minimal fiber Caf1M–Caf1′–Caf1″ (ternary complex) was an important step in understanding the general principles of subunit assembly via the CU pathway. This structure revealed the fiber conformation of the organelle subunit (Caf1″) and subunit–subunit interactions in fibers [17, 18]. The structure of Caf1M and the chaperone-bound Caf1′ subunit is virtually the same as in the Caf1M–Caf1 binary preassembling complex. However, in contrast to the disordered N-terminal region of Caf1 in binary complex, the N-terminal region of Caf1′ is ordered and forms an antiparallel donor β-strand interaction with the last (F) β-strand of the chaperone-free Caf1″ subunit (Fig. 2, *B*). The donated strand produces a bona fide immunoglobulin-like

topology in the fiber subunit. The N-terminal donor strand was denoted as 'Gd' (d for donor) because it plays the same structural role in the fiber as it does in the (C-terminal) G strand of the canonical immunoglobulin fold [17]. Thus, the release of the subunit from the chaperone–subunit complex and its incorporation into a growing fiber involves an exchange of G1 and A1 donor strands of the chaperone to the Gd strand of the neighboring subunit in the fiber. The replacement of the G1 strand by the Gd strand also involves a change of direction of the donor strand from parallel to antiparallel to the F β-strand of the subunit. This process was predicted earlier for FGS chaperone-assembled adhesive pili [63, 64] and for FGL chaperone-assembled polyadhesins [16] and was termed 'donor-strand exchange'. A similar 'topological transition' [65] was also observed for the P pilus subunit PapE bound to a peptide designed to have the sequence of the proposed donor strand of the PapK subunit, suggesting that the donor-strand exchange takes place during assembly of both types of the organelles.

Chaperones preserve Folding Energy of Subunits for Driving of the Fiber Assembly

No energy input from external sources is required to convert periplasmic chaperone–subunit preassembly complexes to free chaperone and secreted fibers [70], in spite of a much more extensive interface between a chaperone and a subunit than that between fiber subunits [17]. Some clues as to how the process can be energetically driven have been provided by structural studies [17, 18, 65, 67]. Comparison of a chaperone complemented (Caf1′) with a fiber subunit (Caf1″) revealed a large conformational difference [17, 18]. The fiber conformation was referred to as the 'closed' or the 'condensed' conformation [18]. The observed difference between open and closed conformations, involving a rearrangement and condensation of the subunit hydrophobic core, suggested that periplasmic chaperones might trap subunits in a high-energy molten globule-like folding intermediate state [17]. A model was proposed in which release of the subunit, followed by Gd donor-strand complementation, allows folding to be completed, driving fiber formation [17]. In contrast to the bulky hydrophobic donor residues in the chaperone G1 donor strand, many smaller donor residues in the subunit N-terminal Gd donor segment do not intercalate between the two sheets of the subunit β-sandwich, allowing close contact between the two sheets [17, 18].

A significant stabilizing contribution from the final fine packing of the hydrophobic core of the subunit is suggested by the melting of the native ternary complex. Structurally observed complete collapse of the Gd-complemented fiber Caf1″ subunit results in a dramatic increase in enthalpy and transition temperature for melting the fiber module. Thermodynamic studies provide strong evidence for the hypothesis that collapse of the subunit hydrophobic core shifts the equilibrium toward fiber formation [18].

Recently, the mutagenesis of the binding motifs of the Caf1M chaperone and Caf1 capsular subunit was performed and analyzed the effect of the mutations on the structure, stability, and kinetics of Caf1M–Caf1 and Caf1–Caf1 interactions [32]. The results suggest that a large hydrophobic effect combined with extensive main-chain hydrogen bonding enables Caf1M to rapidly bind an early folding intermediate of Caf1 and direct its partial folding. The switch from the Caf1M–Caf1 contact to the less hydrophobic, but considerably tighter and less dynamic Caf1–Caf1 contact occurs via the zip-out–zip-in donor strand exchange pathway with pocket 5 acting as the initiation site.

MOLECULAR ARCHITECTURE OF ADHESINS

The final architecture and morphology of linear fibers depend on the subunit composition and the mode of subunit–subunit interactions. These factors determine a coiling of secreted linear fibers into different structures. Fig. 3, *A* shows the scheme of coiling of FGS chaperone-assembled thick rigid monoadhesive pili with a diameter of 7–8 nm. The data on the architecture of monoadhesins were reviewed by [11–15, 71–76]. The scheme of FGS chaperone-assembled thin flexible heteropolyadhesins with a diameter of 2–4 nm is shown in Fig. 3, *B*. The data on the architecture of FGS chaperone-assembled heteropolyadhesins were reviewed by [77]. The scheme of FGS chaperone-assembled homopolyadhesins with a diameter of about 2 nm is shown in Fig. 3, *C* [51]. The structures of FGL chaperone-assembled polyadhesins with a diameter of about 2 nm are shown in the Fig. 3, *D* and *E* [42, 78]. Fig. 3, *F* and *G* show the structures of alternative chaperone-assembled polyadhesin ECP [6], composed of two sequentially combined polyadhesive homopolymers of EcpA and EcpD subunits, respectively (Fig. 3, *F*), with a dual role in biofilm formation

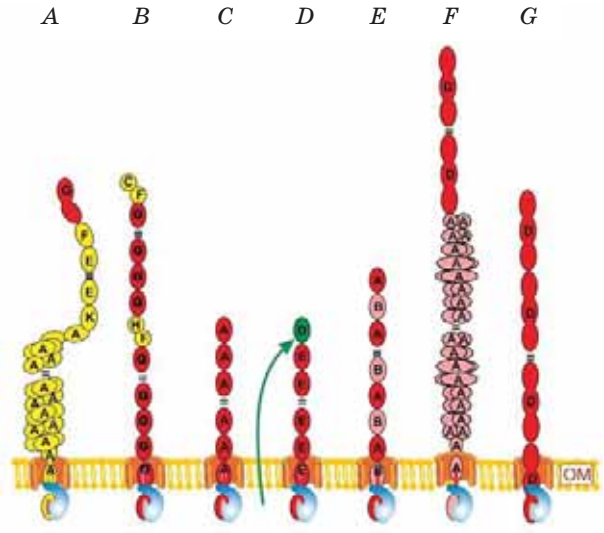

Fig. 3. A — **Schematic presentation (elaborated by the authors) of the structure of FGS chaperone-assembled thick rigid monoadhesive fimbriae/pili (P pili as example) [75, 76];** ***B*** **— FGS chaperone-assembled hetero-polyadhesins (F4, K88 pili as example) [77];** ***C*** **— FGS chaperone-assembled homo-polyadhesins (PE fimbriae as example) [51];** ***D, E*** **— FGL chaperone-assembled polyadhesins (*D* — AfaE polyadhesin as example [42];** ***E*** **— CS6 heteropolyadhesin as example [78]);** ***F, G*** **— alternative chaperone-assembled polyadhesin ECP [6] composed of two sequentially combined polyadhesive homopolymers of EcpA and EcpD subunits, respectively (*F*), with a dual role in biofilm formation and host cell recognition or polyadhesive homopolymer of EcpD subunits (*G*) with a separate role in host cell recognition.**

Periplasmic chaperones and outer membrane ushers are in blue and light orange, respectively. Adhesin subunits are in red. Structural subunits are in yellow. Green arrow shows chaperone/usher-independent secretion of AfaD subunit (shown in green) via type II secretion system [46] and its potential display on the tip of the AfaE fimbrial polyadhesin [42]

and host cell recognition and polyadhesive homopolymer of EcpD subunits (Fig. 3, *G*) with a separate role in host cell recognition. The scanning electron microscopy images show that the pili of EcpA subunits are thin flexible fibers that extend several micrometers away from the bacterial surface and have a high tendency to aggregate into well-ordered parallel and 12-nm antiparallel superstructures, implicating an important role for EcpA in biofilm formation [6]. The FGL chaperone-assembled polyadhesins can aggregate to form amorphous masses or capsules, for example the F1 capsular antigen [79], NFA-I [39], NFA-I-like Dr-II [80] or afimbrial adhesins III, VII and VIII [40, 81].

In the case of FGS chaperone-assembled monoadhesive fimbriae/pili, the specialized adhesive subunit always occurs at the tip of fimbriae, either as the distal end of thin (~2.5 nm) and flexible fimbriae (e.g. F17G from F17 fimbriae) or at the edge of a thin (~2.5 nm) tip fibrillum that is stuck onto a relatively rigid, 1–2-mm-long and ~7.5-nm-wide right-handed helical pilus rod (e.g. PapG of P pili and FimH of type I pili) (Fig. 3, *A*) [82]. This specialized subunit is called an adhesin. All adhesive subunits of monoadhesive fimbriae/pili are two-domain adhesins [63, 82–86]. A two-domain adhesion consists of an N-terminal receptor-binding domain that can be stably expressed on its own and a rather conserved C-terminal pilin domain. Both domains have an immunoglobulin-like fold and are joined via a short interdomain linker. The few known crystal structures of tip-located receptor-binding N-terminal adhesin domains of monoadhesive fimbriae/pili, PapGII, FimH and F17G/GafD, show that, despite little or no sequence identity, common to them all is an elongated β-barrel jelly-roll fold that contains the receptor-binding groove [63, 82–86]. The adhesin domains differ in disulfide patterns, the size and location of the ligand-binding groove, as well as in the mechanism of receptor binding. In particular, their glycan-binding sites have evolved in different locations onto this similar scaffold, and with distinct, highly specific binding properties.

Subunits of monoadhesive fimbriae are called pilins. In particular, P fimbriae are composed of ~1000 copies of the major subunit protein PapA, which polymerize to form a rigid stalk connected to a flexible tip consisting of limited copies of the minor subunit proteins PapE and PapF and receptor-binding adhesin PapG at the distal end [87, 88]. Type I pili are composed of up to 3000 copies of the subunit FimA, which form a stiff, helical pilus rod, and subunits FimF, FimG and FimH, which form the linear tip fibrillum. All subunits in the pilus interact via the donor-strand complementation, in which the incomplete immunoglobulin-like fold of each subunit is complemented by insertion of an N-terminal extension from the following subunit.

In the case of FGL chaperone-assembled polyadhesins, all subunits may possess two independent binding sites specific to different host-cell receptors [42, 43, 46, 47, 89–91]. Dimensions of the bacterial polyadhesive fibers Dr, whose assembly is assisted by the FGL chaperone, were investigated with negative-stain electron microscopy [42]. Thin flexible fibers (2 nm diameter) were observed. The results are entirely consistent with the model with end-to-end contact between each subunit (Fig. 5) [42] and are reminiscent of the model

of capsular F1 antigen from *Y. pestis*, Caf1 [17]. Similar thin fibers have been observed for the pH6 antigen [33]. In addition to the predominance of thin fibers Dr, the electron microscopy also revealed a thicker morphology with overall dimensions larger than the linear model suggested [42]. Thick fibers are not consistent with end-to-end contact and imply that more extensive intersubunit interactions also exist. This would rigidify the resulting rod by the tighter coiling of a single fiber or formation of a trimeric coiled-coil arrangement of fibers.

The ultrastructure of the *Y. pestis* capsule was examined with whole bacteria and negative stain transmission electron microscopy [92]. Bacteria were grown to the logarithmic phase at 37 °C, pH 7.4. The appearance of the capsule was more clearly visible than reported in previous studies, in which the capsule generally appeared as an amorphous haze or as a dense mass surrounding the bacteria [79, 93, 94]. The *Y. pestis* KIM61 strain consistently produced an extended halo composed of thin fibrils and denser aggregates. This denser capsular material, likely composed of aggregates of the thin fibrils, sometimes extended out from the bacterial surface in long strands. The thin, fibrillar appearance of the F1 capsule resembles structures previously reported for other members of the FGL family of chaperone/usher pathways, including the pH6 antigen of *Yersinia* [33, 36] and the CS3 and CS6 pili of ETEC [95, 96]. The negative-stain electron microscopy and single-particle image analysis was used to determine the 3D structure of the *S. typhimurium* Saf polyadhesin [97]. The Saf polyadhesin comprises highly flexible linear multisubunit fibers that are formed by globular subunits connected to each other by short links, giving a 'beads on a string'-like appearance. Quantitative fitting of the atomic structure of the SafA polyadhesin subunit into the electron density maps, in combination with linker modeling and energy minimization, has enabled analysis of subunit arrangement and intersubunit interactions in the Saf polyadhesin. Short intersubunit linker regions provide themeans for flexibility of the Saf polyadhesin by acting as molecular hinges that allow a large range of movement between consecutive subunits in the fiber.

FUCTIONS OF POLYADHESINS

Common Features of Anti-Immune and Proinflammatory Activities of Polyadhesins

In contrast to mono-adhesive pili, which possess only one binding domain on the tip of the pilus (Fig. 4, *A*), each poly-adhesive fiber potentially might (Fig. 4, *B*) [1, 2, 8]:

- ensure a powerful polyvalent fastening of a bacterial pathogen to a host target cell;
- aggregate host-cell receptors and trigger transduction of signals, causing immunosuppressive and proinflammatory responses;
- pull a bacterium to a host cell by a zipper-like mechanism that increases tightness of the contact.

A tight contact between interacting cells hampers diffusion of Ca^{2+} in the site of contact and consequently triggers the Ca^{2+}-dependent type III secretion system (encoded by the pCD1 virulence plasmid) that destroys the defense activity of the host cell [98–101]. This is extremely important for bacterial virulence. In particular, *Y. pestis* appears to utilize the type III secretion pathway to destroy cells with

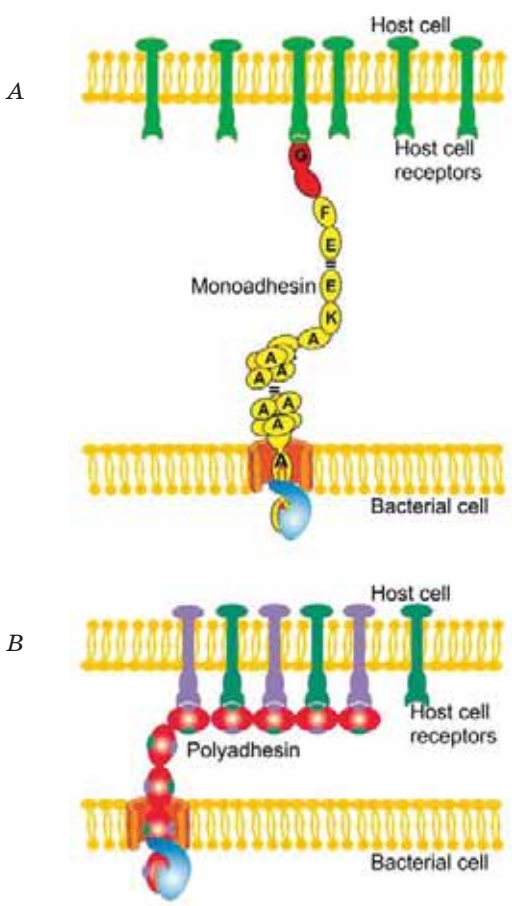

Fig. 4. **Schematic illustration (elaborated by the author) of a binding of monoadhesins (*A*) and polyadhesins (*B*) to host-cell receptors.**
Periplasmic chaperones and outer membrane ushers are in blue and orange, respectively. Adhesin and pilin subunits are in red and yellow, respectively. Host receptors for monoadhesins are shown in green. Two different types of host receptors for polyadhesins are shown by violet and greencyan

innate immune functions (macrophages, dendritic cells and neutrophils), which represent the first line of defence thereby preventing adaptive responses and precipitating the fatal outcome of plague [102]. It was found that dendritic cells infected with *Y. pestis* failed to adhere to solid surfaces and to migrate toward the chemokine CCL19 in an *in vitro* transmembrane assay. Both effects were dependent on the presence of a pCD1 plasmid, and on bacterial growth shift to 37 °C, before infection [103].

It was found that *Y. pestis* adhesins facilitate Yop delivery to eukaryotic cells and contribute to plague virulence [104]. The Ail, plasminogen activator (Pla) and pH6 antigen (Psa) could mediate Yop translocation to host cells. The contribution of each adhesin to binding and Yop delivery was dependent upon the growth conditions. When compared to an *ail* mutant, additional deletion of *psaA* (encoding Psa) led to a 130,000-fold increase in LD_{50} in mice relative to the KIM5 parental strain. These results indicate that Psa can serve as environmentally-specific adhesin to facilitate Yop secretion, a critical virulence function of *Y. pestis*.

Binding of Polyadhesins to Host-Cell Receptors

Afa/Dr polyadhesins — Afa/Dr polyadhesins Dr, F1845 (DaaE), NFA-I and AfaE-III adhesins allow binding to the Dra blood-group antigen presented on the CD55/decay-accelerating factor (DAF), a complement-regulatory and signaling molecule [105]. Under physiological conditions, CD55/DAF plays a central role in preventing the amplification of the complement cascade on host-cell surfaces [106, 107]. CD55/DAF interacts directly with membrane-bound C3b or C4b and prevents the subsequent uptake of C2 and factor B.

The residues of AfaE-III adhesion involved in CD55/DAF binding were localized (Fig. 5) [42]. The binding regions for AfaE-III and the complement pathway convertases lie in close proximity to each other on CD55/DAF. Binding of adhesin Dr to CD55/DAF is inhibited by chloramphenicol, whereas binding of AfaE-III is unaffected [105, 108]. This was used to locate the DraE adhesive site. The 3D structure of the strand-swapped trimer of wild-type DraE in complex with chloramphenicol was solved. NMR data supported the binding position of chloramphenicol within the crystal [43, 44]. Chloramphenicol binds to a surface pocket between the N-terminal portion of strand B and the C-terminal portion of strand E and lies within the recently identified CD55/DAF-binding site (Fig. 5) [42].

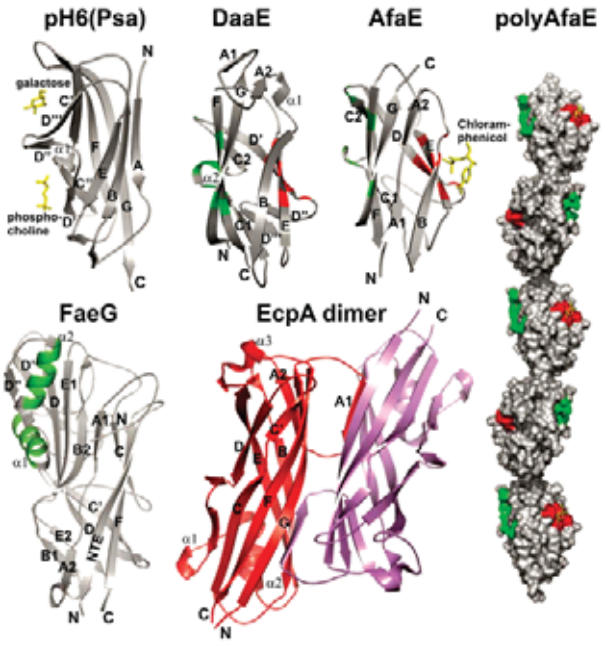

Fig. 5. **Binding sites of FGL chaperone-assembled polyadhesin subunits DaaE and AfaE, FGS chaperone-assembled polyadhesin subunit FaeG and alternative chaperone-assembled polyadhesin subunit EcpA.**

Ribbon diagrams presentations of the DaaE subunit of a strand-swapped trimer of wild-type DaaE of F1845 adhesin and a self-complemented AfaE subunit of AFA-III adhesin with chloramphenicol as a yellow stick presentation. CD55/DAF- and CEACAMS-binding sites derived from DraE and DaaE mutagenesis are shown in green and red, respectively. Molecular surface rendering of a model for the AfaE fiber was generated by assuming the same orientation between successive subunits as observed for Caf1′ and Caf1″ in the F1 fiber [17]. The residues involved in binding with CD55/DAF and CEACAMS are in green and red, respectively. The binding site in FaeG is shown by green. The redrawing is based on the data and coordinates of atoms of the structures published by [6, 42–44, 55, 89, 90, 117]. The figures were prepared with PYMOL [69]

The 3D structure of DaaE at resolution 1.48E also was solved [90]. Trimers of the protein were found in the crystal, as has been the case for other adhesins Dr. Naturally occurring variants and directed mutations in DaaE have been generated and analyzed for their ability to bind CD55/DAF. Mapping of the mutation sites onto the DaaE molecular structure shows that several of them contribute to a contiguous surface that is likely the primary CD55/DAF-binding site (Fig. 5).

Dr, F1845 (DaaE) and AfaE-III adhesins also interact with carcinoembryonic antigen (CEA)-related cellular adhesion molecules CEACAM1, CEACAM5 and CEACAM6 [109]. This recognition is followed by activation of

CEACAMS-associated signaling by pathogens triggering the cellular events. CEACAM1, CEACAM5 and CEACAM6 belong to the immunoglobulin superfamily of adhesion molecules [110]. CEACAM1 has been shown to be expressed in leukocytes, including granulocytes, activated T cells, B cells and natural killer cells [111]. CEACAM1 acts as a novel class of immunoreceptor tyrosine-based inhibition motif-bearing regulatory molecules on T cells that are active during the early phases of the immune response in mice [112–116].

Random mutagenesis with functional analysis and chemical shift mapping by NMR show a clear-cut CEACAMS binding site located primarily in the A, B, E and D strands of the adhesin Dr subunit (Fig. 5) [89]. This site is located opposite the β-sheet encompassing the previously determined binding site for CD55/DAF, which implies that the polyadhesin Dr can bind simultaneously to both receptors on the epithelial cell surface. The structure of the CEA/Dr adhesin complex was proposed based on NMR spectroscopy and mutagenesis data in combination with biochemical characterization [47, 91]. The Dr adhesin/CEA interface overlaps appreciably with the region responsible for CEA dimerization. Binding kinetics, mutational analysis and spectroscopic examination of CEA dimers suggest that adhesins Dr can dissociate CEA dimers before the binding of monomeric forms [47, 91].

pH 6 (Psa) polyadhesin — It was found by flow cytometry that individual *Y. pestis* cells can express the capsular F1 antigen concomitant with the pH6 antigen (Psa) on their surface when analyzed [94]. Isogenic Δ*caf* (F1 genes), Δ*psa* and Δ*caf*Δ*psa* mutants were constructed and studied with the three respiratory tract epithelial cells. The Δ*psa* mutant bound significantly less to all three epithelial cells compared with the parental wild-type strain and the Δ*caf* and Δ*caf*Δ*psa* mutants, indicating that the pH6 antigen acts as an adhesin for respiratory tract epithelial cells.

It was found that the pH6 antigen of *Y. pestis* is a novel bacterial immunoglobulin G (IgG)-binding receptor [118]. A pseudoimmune complex with human IgG1, IgG2 and IgG3 was formed. No binding to human IgG4, rabbit, mouse or sheep IgG was found. Antigen pH6 binds the human IgG1 Fc subunit and does not bind Fab and pFc' subunits. This finding may be explained by pH6 antigen binding to the β1-linked galactosyl residue [119] in a carbohydrate moiety of human IgG1, IgG2 and IgG3 that is linked to CH_2 domains of their Fc subunit [120].

Purified pH6 antigen selectively binds to apolipoprotein B-containing lipoproteins (low-density lipoproteins) in human plasma [121].

Binding of purified recombinant pH6 antigen to gangliotetraosylceramide, gangliotriaosylceramide and lactosylceramide was indicated by an enzyme-linked immunosorbent assay (ELISA) [119].

It was found that pH6 (Psa) antigen fimbriae mediate bacterial binding to human alveolar epithelial cells [122]. The receptor of the pH6 antigen was identified as phosphatidylcholine. The results indicate that the pH6 antigen uses the phosphorylcholine moiety of phosphatidylcholine as a receptor to mediate bacterial binding to pulmonary surfactant and alveolar epithelial cells [122].

The crystal structure of PsaA in complex with both galactose and phosphocholine reveals separate receptor binding sites that share a common structural motif, thus suggesting a potential interaction between the two sites (Fig. 5) [123]. Mutagenesis of this shared structural motif identified Tyr126, which is part of the choline-binding consensus sequence but is found in direct contact with the galactose in the structure of PsaA, important for both receptor binding. Thus, this structure depicts a fimbrial subunit that forms a polymeric adhesin with a unique arrangement of dual receptor binding sites.

F4 (K88) polyadhesin — Enterotoxigenic *E. coli* expressing F4 (K88) fimbriae are the major cause of porcine colibacillosis and are responsible for significant death and morbidity in neonatal and postweaned piglets. F4 fimbriae are assembled into thin, flexible polymers mainly composed of the single-domain adhesin FaeG [55, 77, 117]. FaeG has an Ig core made up of strands A1, A2, B1, B2, C, D, E1, E2, F and G, named according to their place in the Ig-fold and in analogy to the nomenclature used for other fimbrial subunit structures (Fig. 5) [55, 117]. These show an incomplete Ig-like structure, lacking the last β-strand G. In pili, this G-strand is provided by the Nte of the adjacent subunit. In the Ig fold of the FaeG, a short helical turn occurs between strands A2 and B1, and an extra strand C′ is inserted between strands C and D. In addition to the Ig-folded core, the FaeG structure contains an extra domain introduced between strands D and E1. This domain is composed of strands D′ and D″, linked by two α-helices, α1 and α2.

The receptor binding site on FaeG was described as a spatial arrangement of two amino acid residues with a hydrophobic side-chain

(Phe/Leu134 and Phe/Leu/Met147) in combination with one or more amino acid residues with hydrophilic and charged side-chains (Lys/Arg136, Arg/Ser/His155 and Asp/Asn216, or in the hypervariable region comprised of residues 163–173) [52]. The structure of FaeG allowed to localize the aforementioned residues and the hypervariable regions comprising residues 163–173 and residues 206–216 on the surface of FaeG (Fig. 5, shown by green) [117]. This suggests that the receptor-binding site of FaeG is not located within the Ig core of the protein but rather in the extra domain made up of strands D′ and D″, and α-helices α1 and α2. Especially the long loop between D′ and α1 and the loop connecting the extra domain to the Ig core (between D″ and E1) are indicated as being part of the binding site.

ECP (Mat) polyadhesin — First data on a novel fimbria isolated at low temperatures from *E. coli* associated with newborn meningitis and septicaemia (NMEC) were published by [124]. This surface organelle was called the meningitis associated and temperature regulated (Mat) fimbria, although later by other groups was shown that this fibrillar organelle is ubiquitous across most *E. coli* strains and it is now usually referred to as the *E. coli* common pilus (ECP) [125–128]. *E. coli* are primarily commensal colonizers of the human and other animal bowels and they contribute to a healthy immune system of the host. There are also a number of virulent strains that can cause diarrheal diseases such as hemorrhagic colitis [129]. Furthermore, if they enter extraintestinal sites these strains can also lead to neonatal meningitis, urinary tract infections, sepsis, and pneumonia [130].

ECP fibres are assembled via the alternative CU pathway and the organelles are formed from polymerization of two pilin subunits (Fig. 3, *F* and *G*) [6]. The tip of ECP is uniquely composed from a polymerized array of a novel adhesive subunit EcpD (~ 60 kDa) recognizing an unknown ligand on the host cell surface [6]. The majority of ECP is composed of an 18 kDa domain called EcpA [124, 125], which functions in binding hydrophobic surfaces and mediating interbacterial aggregation in early biofilm formation [6, 131].

The crystal structure of EcpA from uropathogenic *E. coli* has been recently solved (Fig. 5; PDB: 3QS2, 3QS3) [6]. Like other CU major pilin domains, EcpA is formed from an incomplete Ig-like fold, where an adjacent molecule in the fiber donates its N-terminal strand (N-terminal extension) to fill a hydrophobic groove running along the full length of EcpA, completing the very stable Ig-like motif (Fig. 5). EcpA is fashioned from approximately 50% hydrophobic residues and the surface is scattered with hydrophobic patches including a number of aromatic residues. This likely promotes a less-specific contact with a wide range of hydrophobic substrates and polymers.

ECP are quite flexible with a width ~6 nm, which consistently varies along the fibre length (Fig. 3, *F*) [6]. Electron microscopy images of *E. coli* producing ECP show these fibres form a mesh that encapsulates the whole microcolony. ECP interacts with itself through pili crossing over one another, parallel fibre entwining and antiparallel entwining [6]. The crystal lattice of EcpA also revealed an intertwining of antiparallel fibres giving rise to a super helical diameter of ~12 nm. EcpA is highly conserved amongst a range of other enteric bacterial species including *Serratia proteamaculans*, *Serratia odorifera*, *Klebsiella sp.*, *Klebsiella pneumoniae*, and *Enterobacter cancerogenus*, which suggests a role for ECP in establishing contacts between multiple species [6].

CONCLUSIONS AND FUTURE PERSPECTIVES

Among the bacterial causes, enterotoxigenic *E. coli* (ETEC), *E. coli* strains producing enterotoxins, are the most common cause of diarrheal disease in children living in endemic areas, as well as children and adults traveling to these areas. Approximately 280–400 million ETEC-associated diarrhea cases occur annually in children younger than 5 years old, plus 100 million more cases in children older than 5 years, which results in approximately 300,000–500,000 deaths annually [132]. Travelers are also susceptible to diarrhea as they are usually immunologically naive and are exposed to the environment in developing countries. Among the causes of traveler's diarrhea, ETEC is the most common. It was estimated that 400 million adult diarrheal cases are associated with ETEC [132].

After half a century of highly productive antibiotic development, however, it has now become obvious that antibiotics cannot provide the ultimate solution in the fight against bacterial infections. The non-critical use of antibiotics in human and veterinary medicine has caused widespread resistance in bacteria. Antibiotic-resistant strains of Gram-negative pathogens have emerged extensively in the

last dozen years, whereas safe and effective vaccines against many of them are currently not available. There are now a growing number of reports of cases of infections caused by Gram-negative organisms for which no adequate therapeutic options exist [133, 134]. This return to the preantibiotic era has become a reality in Europe as well as in other parts of the world. A large outbreak of diarrhea and the hemolytic-uremic syndrome caused by an unusual serotype of Shiga-toxin-producing *E. coli* (O104:H4) occured in Germany in 2011. A large number of cases of diarrhea caused by Shiga-toxin-producing *E. coli* (STEC) have been reported — 3167 without the hemolytic-uremic syndrome (16 deaths) and 908 with the hemolytic-uremic syndrome (34 deaths) — indicating that this strain is notably more virulent than most of the STEC strains [135]. The pAA plasmid of C277-11 (referred to here as pAA C277-11) encodes the *agg* cluster responsible for expression of the aggregative adherence fimbriae Type I, AAF-I. The AAF-I fimbriae are related to the family of polyadhesins [17]. The main subunit of the AAF-I fimbriae, AggA, together with the additional subunit AggB may be used for development of vaccine against this STEC strain. Among the most common protective antigens for design of vaccines against ETEC are the CS3 and CS6 polyadhesins [132].

Passive immunization has recently become an even more attractive approach because of individuals with impaired immune systems who are unable to respond to conventional vaccines. Also, passively administered antibodies have the ability to provide rapid and immediate protection. Anti-bacterial serum therapy is now being reinvented in modern biotechnology terms, in the form of monoclonal antibodies [10]. However, when considering antibody production for passive immunotherapy applications for treatment of enteric bacterial infections, chickens present a much more economical source of large quantities of specific antibodies [9]. Chiken IgY yields have been reported to range from 60 to 150 mg IgY per egg. Given that a typical hen can lay approximately 325 eggs per year, this can result in a potential yield of around 20–40 g of IgY per year, of which 2% to 10% is antigen-specific [9].

IgY has been tested against a number of enteric pathogens. IgY produced against the porcine ETEC polyadhesins F4 (K88) and F5 (K99) was found to inhibit the binding of *E. coli* K88-, K99-positive strains to porcine epithelial cells and porcine intestinal mucus *in vitro* [9]. When given orally to piglets, these antibodies dose-dependently protected against *E. coli* infection. It was found that when anti-$K88^+$ ETEC IgY was encapsulated in chitosan-alginate microparticles, it exerted its anti-diarrheal effects much faster (24 h versus 72 h postinfection in pigs given nonencapsulated IgY) and led to increased weight gain when compared to pigs fed nonencapsulated antibodies [9]. The passive protective effect of anti–*E. coli* IgY in cattle has also been shown. Neonatal calves fed milk containing anti-ETEC IgY had transient diarrhea, 100% survival, and improved body weight gain [9].

Thus, the polyadhesins are potential targets for immune countermeasures against bacterial infections, in particular for anti-adhesion therapy with antibodies to polyadhesins as one of alternatives to antibiotic therapy.

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ПОЛІАДГЕЗИНИ: ЗАСІБ ДЛЯ ПРОНИКНЕННЯ ГРАМНЕГАТИВНИХ ПАТОГЕНІВ КРІЗЬ ІМУННИЙ БАР'ЄР

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Швидка поява резистентних до лікування бактеріальних патогенів стала однією з основних небезпек для здоров'я населення. Нещодавній спалах інфекції нового шига-токсину кишкової палички O104H4 в Німеччині ілюструє цю проблему. Для колонізації тканин хазяїна патогенні бактерії експресують поверхнево-адгезивні органели. Німецький штам використовує агрегативний імунний адгезив *fimbriae I* для прикріплення до слизової оболонки кишечника, спричинюючи запалення. *Fimbriae I* належить до родини шаперонпровідних асоційованих фімбріальних поліадгезинів, що функціонують як механізм проникнення крізь імунний бар'єр хазяїна. Зв'язування поліадгезинів із клітинами-мішенями координується інфектисомою (третя система секреції), що вкрай важливо для вияву бактеріальної вірулентності і сприяє індукції деструктивних сигналів агрегації рецепторів клітини-хазяїна, які дають змогу патогенам ввести в клітину хибну інформацію та проникнути через імунний бар'єр. Поліадгезини також залучені в утворення біоплівки, що робить бактерії стійкішими до імунної відповіді. З огляду на це поліадгезини є потенційними мішенями для розроблення контрметодів імунного захисту проти бактерійних інфекцій, зокрема антиадгезивної терапії відповідними антитілами як однієї з альтернатив антибіотикотерапії.

Ключові слова: грамнегативні патогени, поліадгезини, подолання імунного бар'єра.

ПОЛИАДГЕЗИНЫ: СРЕДСТВО ДЛЯ ПРОНИКНОВЕНИЯ ГРАММОТРИЦАТЕЛЬНЫХ ПАТОГЕНОВ ЧЕРЕЗ ИММУННЫЙ БАРЬЕР

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Быстрое появление резистентных к лечению бактериальных патогенов стало одной из основных опасностей для здоровья населения. Недавняя вспышка инфекции нового шига-токсина кишечной палочки O104H4 в Германии иллюстрирует эту проблему. Для колонизации тканей хозяина патогенные бактерии экспрессируют поверхностно-адгезивные органеллы. Немецкий штамм использует агрегативный иммунный адгезив *fimbriae I* для прикрепления к слизистой оболочке кишечника, вызывая воспаление. *Fimbriae I* принадлежит к семейству шаперонпроводящих ассоциированных фимбриальных полиадгезинов, которые функционируют в качестве механизма проникновения через иммунный барьер хозяина. Связывание полиадгезинов с клетками-мишенями координируется инфектисомой (третья система секреции), что крайне важно для проявления бактериальной вирулентности и способствует индукции деструктивных сигналов агрегации рецепторов клетки-хозяина, позволяющих патогенам ввести в клетку ложную информацию и проникнуть через иммунный барьер. Полиадгезины также вовлечены в образование биопленки, которая делает бактерии более устойчивыми к иммунному ответу. Поэтому полиадгезины являются потенциальными мишенями для разработки контрметодов иммунной защиты против бактерийных инфекций, в частности антиадгезивной терапии соответствующими антителами как одной из альтернатив антибиотикотерапии.

Ключевые слова: граммотрицательные патогены, полиадгезины, преодоление иммунного барьера.

UDK 578.27+615.32

THE GMP-BASED DRUG SUBSTANCE SCTL DEVELOPMENT AIMING AT PREVENTION OF OPPORTUNISTIC INFECTIONS AFTER X-RAY- AND CHEMOTHERAPY OF CANCER. A SYNTHETIC COMBINATORIAL TETRAPEPTIDE LIBRARY SUBSTITUTION FOR CALF THYMUS EXTRACT

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SCTL is the fully synthetic correlate of an enzymatic partially hydrolyzed extract from calf thymus. To exclude completely the transmission of bovine spongiformic encephalitis by the bovine thymus product a fully synthetic correlate of the active principles in the thymus tissue hydrolysate has been developed, namely SCTL. This synthetic peptide library has meanwhile substituted calf thymus extract preparations in several cosmetics and drug products. The active principles of SCTL have been invented by the author but the application of the drug substance in cosmetic and pharmaceutical products has been exploited by others. For SCTL only limited pharmacological and toxicological data are available. Some interesting biological activities, though, have been shown for SCTL which might explain to some extend the modes of action and its clinical effectiveness.

Key words: synthetic correlate of an enzymatic partially hydrolyzed extract from calf thymus.

SCTL is the fully synthetic correlate of an enzymatic partially hydrolyzed extract from calf thymus, HTX. To exclude completely the transmission of bovine spongiformic encephalitis (BSE) by the bovine thymus product a fully synthetic correlate of the active principles in the thymus tissue hydrolysate has been developed, namely SCTL. This synthetic peptide library has meanwhile substituted calf thymus extract preparations in several cosmetics and drug products.

The active principles of SCTL have been invented by the author but the application of the drug substance in cosmetic and pharmaceutical products has been exploited by others. For SCTL only limited pharmacological and toxicological data are available. Some interesting biological activities, though, have been shown for SCTL [Birr et al., 1987, 1998, 2003] which might explain to some extend the modes of action and its clinical effectiveness.

In traditional European medicine the application calf thymus extract preparations in geriatric and immunodeficiency diseases has been practised for more than three centuries. In several countries traditional drug preparations and cosmetics from HTX have been used successfully. There are field reports on prevention of alopecia, opportunistic infections and cancer. Several publications report about the efficacy of the product in the reduction of hair loss secondary to cytostatic chemotherapy.

But the mode of action of HTX , the partially hydrolyzed extract from calf thymus, is not known. Since biological effects on the pro-

*)With appreciation dedicated to Professor Serhiy V. Komisarenko, Palladin Institute of Biochemistry, Kyiv, Ukraine, on the occasion of his 70th birthday, July 9, 2013.

liferation of human lymphocytes have been shown, actions similar to other thymus proteins are assumed. Precise descriptions of the mode of action are hampered by the fact that drug products from HTX are by no means consistent in preparation and composition.

In the effort to completely prevent the transmission of BSE by this kind of product a fully synthetic peptide library SCTL was developed which resembles the major components of HTX in its chemical composition. SCTL contains di-, tri-, tetrapeptides and free amino acids, all in number and quantity specific for calf thymus partial hydrolysates. The preparative consistency and chemical composition of this well-defined synthetic drug product will be described in more detail in the next section of this article.

Chemistry of SCTL

Background

The European bovine & transmissible spongiformic encephalitis (BSE / TSE) catastrophe of the recent decades has put this entire field of natural bovine extract therapeutics into crisis. Many thymus preparations have become banned by law. Since then, a very well established clientele among the elderly, but also patients, therapists, pharmacies and industries were looking for safe alternatives for many of these well established bovine tissue extract drug products.

One of these products was HTX from bovine starting material. The risk of BSE/TSE transmission could not be fully excluded. Even if the traditional manufacturing process was carried out thoroughly, due to the complex composition (amino acids, peptides, saccharides, fats etc.) and the naturally occurring variability in the primary tissue source, an undesirable inconsistency in the product properties was observed. Due to the lack of a specific chemical marker, it was not possible to compensate this by standardising the product at the end of the manufacturing process. Owing to the manufacturing process varying by-products from thymus tissue, which were considered to be impurities remained in the finished. It was not possible to separate these from the target fraction of the enzymatic hydrolysate.

A major research & development project was launched at the author's laboratory by an industry sponsor to find a composition which has similar pharmacological properties and is comparable to the main fraction of the partial enzymatic hydroly-sate HTX. In order to achieve the closest synthetic version, analytical investigations were carried out with emphasis on the peptide composition of this natural tissue extract. The aim of these studies was the development of a fully synthetic chemically standardized product resembling as close as possible the chemical composition and the immune-pharmacological properties of HTX.

The main fraction of HTX has an 80% protein content and a molecular weight range of up to 10 kDa. It was determined that one third of the natural material contains free amino acids together with short oligo peptides, which by pool sequence analysis were determined to consist mainly of tetrapeptides accompanied by traces of di- and tripeptides. Furthermore, there were some other natural compounds like hexoses, saccharides, sialic and nucleic acids, also modifications of the peptide compositions were detected.

Based on these results it was considered to synthesize a statistic combinatorial peptide library composed out of di-, tri- and tetrapeptides and a pool of free amino acids, in number and molar proportional quantity similar to the amino acid composition of

the main fraction of HTX, without adding any further ingredient. Following this way SCTL has been developed as a synthetic version of the peptide content in natural thymus partial hydrolysate, conceptually differing from the development of molecularly defined thymic polypeptides [Birr et.al., 1979, 1983, 1984].

Analytical Investigations

Due to the inconsistency in the composition of HTX several batches of the natural product were analysed for obtaining reliable mean values.

The sequencing of the terminal amino acids of the main fraction of HTX resulted in a termination after three cycles, leading to the conclusion that the natural hydrolysate consists mainly of free amino acids as well as di-, tri- and tetrapeptides. The sequencing also showed that these peptides consist of a statistical distribution of homologues which could not be further separated for component identification.

The analytical data show that HTX consists of approximately 32.5% free amino acids, the remaining are di-, tri- and tetrapeptides. The peptides consist mainly of Asp, Glu, Pro, Gly, Ala, Val, Leu, Lys and Arg, whereas the absence of Cys in the peptides may result from oxidative destruction during analysis .

The lipid amount was about 8% and the amount of nucleic acids less than 0.1% .

The amount of other compounds found were 8% hexoses, up to 1.5% sialic acid and

about 5% mono saccharides. These compounds of HTX were considered as carrier agents or impurities and consequently not included in the peptide synthesis concept for the generation of SCTL.

Analytical comparison of the natural HTX with synthetic SCTL by amino acids analysis and RP-HPLC

A comparison of free and total amino acids is given in Table 1 to Table 3.

The HPLC analysis of synthetic SCTL and the main fraction of the partial hydrolysate of calf thymus HTX show a similar profile as demonstrated in Fig. 1 and Fig. 2.

From these findings it was decided to synthesize a statistic combinatorial peptide library consisting mainly of tetrapeptides and amino acids together with amounts of up to 10% di- and tripeptides each. The pool of amino acids was standardised with regard to the unprotected amino acids in each reaction step.

Studies for determination of the biological activity by mitogen costimulation of separated HTX by-product fractions gave no evidence, that these portions of HTX have any immunological action. Therefore, these were considered to be impurities not required in the composition of the synthetic version.

The synthetic chemical product SCTL has no risk for BSE/TSE transmission as could be the case for natural HTX due to the route of manufacturing of the natural product from bovine thymus tissue. Moreover, the synthetic SCTL does not contain impurities like nuleic acids, sugars and fats, as it is caused for the natural product HTX through its route of manufacture from thymus tissue.

Table 1. **Free amino acids determination in HTX and in SCTL for comparison**

Batch	8863 (1)	8863 (2)	968	T.E.P.	Mean HTX	Theor. SCTL
Amino acid	**[% w/w]**	**[% w/w]**	**[% w/w]**	**[% w/w]**	**[% w/w]**	**[% w/w]**
Asp	2.09	2.15	2.23	3.21	2.42	2.40
Thr	5.70	5.69	4.48	4.67	5.13	9.24[a)]
Ser	3.84	3.82	5.48	4.53	4.42	8.60[a)]
Glu	8.60	8.32	9.78	8.39	8.77	6.47
Pro	3.10	3.11	2.33	3.06	2.90	2.29
Gly	2.77	2.60	2.94	2.98	2.82	4.93
Ala	8.57	8.43	7.88	8.42	8.32	11.67
Cys	–	–	–	–	–	0.61
Val	5.63	5.69	6.3	8.00	6.40	6.74
Met	2.46	2.52	3.23	5.38	3.40	3.23
Ile	4.62	4.41	5.57	5.93	5.13	4.24
Leu	14.74	14.91	14.45	15.87	14.99	14.12
Tyr	4.89	4.41	5.34	1.30	3.99	0.36
Phe	5.56	5.83	5.71	6.36	5.87	4.26
Trp	–	–	–	–	–	0.00
His	1.32	1.30	–	–	0.65	1.22
Lys	12.78	13.24	12.61	11.00	12.41	10.35
Arg	13.32	13.58	11.67	10.91	12.37	9.27
Total	100	100	100	100	100	100

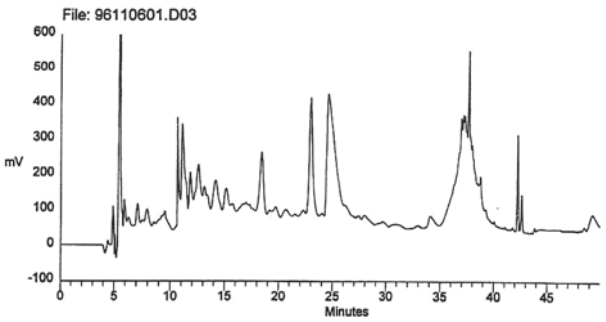


Fig. 1. **RP-HPLC Analysis of synthetic SCTL (batch SP-01)**

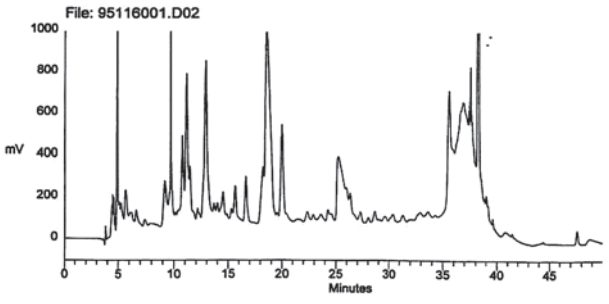


Fig. 2. **RP-HPLC Analysis of natural HTX Reference**

Table 2. **Total amino acid composition in HTX and in SCTL for comparison**

Batch	8863 (1)	8863 (2)	968	T.E.P.	Mean HTX	Theor. SCTL
Amino acid	**[% w/w]**	**[% w/w]**	**[% w/w]**	**[% w/w]**	**[% w/w]**	**[% w/w]**
Asp	8.87	9.20	9.77	7.60	8.86	6.05
Thr	2.83	3.18	3.42	5.19	3.65	3.55
Ser	1.32	1.81	1.85	5.29	2.57	3.30
Glu	14.87	14.94	15.51	14.68	15.00	8.36a)
Pro	6.12	6.01	5.71	7.52	6.34	10.23a)
Gly	10.10	9.47	8.91	10.11	9.65	24.74a)
Ala	8.69	8.40	7.36	7.75	8.05	11.70
Cys	0.00	0.00	0.00	0.58	0.14	0.23
Val	6.44	6.57	6.22	6.28	6.38	6.15
Met	1.44	1.37	1.81	2.56	1.80	1.24
Ile	4.21	4.26	4.63	4.24	4.33	3.53
Leu	8.55	8.67	8.85	7.67	8.44	5.52
Tyr	0.48	0.39	0.91	0.00	0.44	0.14
Phe	3.64	3.60	4.05	3.66	3.74	2.07
Trp	0.00	0.00		0	0.00	0.00
His	1.13	1.08	1.04	0.43	0.92	0.47
Lys	11.90	11.34	11.07	8.49	10.70	6.94
Arg	9.41	9.70	8.89	7.96	8.99	5.77
Total	100	100	100	100	100	100

Table 3. **Free and total amino acid differentiation in HTX and SCTL for determination of the peptide contribution**

Batch	Mean HTX free AA	Mean SCTL free AA	Mean HTX total AA	Mean SCTL total AA	Δ HTX vs. SCTL free AA	Δ HTX vs. SCTL total AA
AA	**[% w/w]**	**[% w/w]**	**[% w/w]**	**[% w/w]**	**[% w/w]**	**[% w/w]**
Asp	2.42	3,11	8,86	6,18	−0,69	2,68
Thr	5.13	6.97[d)]	3,65	2,86	−1,84	0.80
Ser	4.42	5.06[d)]	2,57	2,09	−0,64	0.47
Glu	8.77	6.87	15.00	8,60	1.90	6.40
Pro	2.90	2.67	6.34	8.12[c)]	0.23	−1.78
Gly	2.82	5.60	9.65	33.45[c)]	−2.78	−23.81
Ala	8.32	11.55	8.05	11.21[c)]	−3.22	−3.16
Cys[a)]	0.00	0.05	0.14	0.09	−0.05	0.06
Val	6.40	7.24	6.38	4.88	−0.84	1.50
Met	3.40	3.51	1.80	0.44	−0.11	1.36
Ile	5.13	5.10	4.33	2.91	0.03	1.43
Leu	14.99	15.06	8.44	5.42	−0.07	3.02
Tyr	3.99[b)]	0.13	0.44	0.01	3.85	0.43
Phe	5.87	4.83	3.74	1.98	1.04	1.76
Trp	0.00	0.00	0.00	0.00	0.00	0.00
His	0.65	0.88	0.92	0.20	−0.22	0.72
Lys	12.41	11.40	10.70	6.98	1.01	3.72
Arg	12.37	9.98	8.99	4.59	2.39	4.40
Total	100.00	100.00	100.00	100.00	°	°

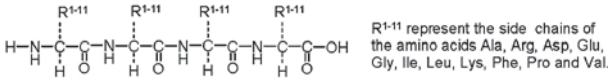
[a)] Destroyed by analysis conditions; [b)] Only 10 % value specified [c)] Surplus for compensation of 0.5 % collagen (Pro, Gly, Ala) in total composition; [d)] Surplus for thermal destruction compensation in total composition.

The Synthetic Combinatorial Thymus Tetrapeptide Library SCTL

The novel drug substance SCTL is a synthetic version of the former natural thymus partial hydrolysate HTX, originally prepared by enzymatic processing from calf thymus tissue. SCTL, mimicking the complex structure of the natural HTX, by synthetic means is composed of amino acids, di-, tri- and tetrapeptides in quantities and nature resembling the composition of partially hydrolyzed thymus tissue.

The drug substance SCTL is a synthetic statistical combinatorial thymus tetrapeptide library composed of:

a) Linear di-, tri- and tetrapeptides containing the residues R1-11 of the natural L-amino acids Alanine, Arginine, Aspartate, Glutamate, Glycine, Isoleucine, Leucine, Lysine, Phenylalanine, Proline and Valine in a statistical combination



and

b) the 17 natural L-amino acids or their HCl salts, respectively: Ala, Asp, Arg×HCl, Cys(H_2O)×HCl, Glu, Gly, His(H_2O)×HCl, Ile, Leu, Lys×HCl, Met, Phe, Pro, Ser, Thr, Tyr and Val.

The Peptide Library SCTL

The synthetic drug substance SCTL is chemically manufactured from and consists only of the naturally occurring L-amino acids, either as free amino acids or as their statistical synthetic combinations in di-, tri- and tetrapeptides and their salts, respectively. These kinds of products are called combinatorial peptide libraries containing the individual amino acids and peptides in all statistically possible combinations. Because of the similarity of the individual components, the molecular quantities in traces of individual peptides in the library cannot be separated from each other and therefore cannot be determined individually. Only the statistical combination of all peptides in the library as a whole can be described and analysed. Therefore, the product SCTL for pharmaceutical development is considered a mono compound drug substance.

The molecular formula given above resembles the statistical combinatorial peptide library of different di-, tri- and tetrapeptides as well as free amino acids. The relative molecular masses have a range from 75 to 643, due to the content of Gly as lowest to a tetrapeptide consisting of $(Arg)_4$ as a maximum. SCTL is an off-white powder, which was manufactured at ORPEGEN, Germany [Birr et al., 1998].

The GMP-based carefully standardized manufacturing process is a six steps operation. In the first, third and fifth step carboxyl protected amino acids, di- and tripeptides, respectively, are coupled with N-protected amino acid derivatives. It was intended to synthesize a synthetic combinatorial peptide library containing trace amounts of monomers, di-, tri- and tetrapeptides similar to the low molecular weight fraction of a partial hydrolysate of calf thymus HTX. This is achieved by the limit of detection (≤ 2%) of the photometric quantitative Ninhydrine method used as an in process control, IPC, carried out during each synthesis cycle of the manufacture. In the second and fourth step the terminal N-protecting groups are removed. In the final step the free peptides are obtained by catalytic hydrogenation of all remaining benzyl type protecting groups.

In all operations, the reactive side groups of those amino acids containing side functions are protected by Z- or OBzl groups, benzyl esters and (Z) benzyloxycarbonyl protection groups, respectively. Some of the protected amino acids are used as salts due to their better solubility and stability. All functional side chains of the natural L-amino acids remain protected during the synthesis up to the last step 6, where they are then deprotected simultaneously together with the N- and C-terminal protecting groups.

The absence of traces from benzyl-type protecting groups was proven by 300 MHz ^{1}H-NMR spectroscopy in seven final drug substance batches of SCTL.

Also by capillary electrophoresis, CE, in a batch-to-batch consistency documentation the synthetic molecular identity of seven dif-

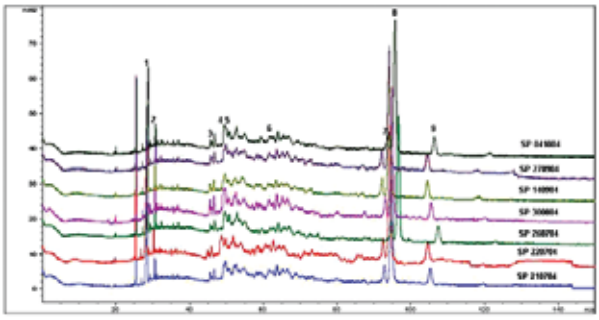


Fig. 3. **Batch-to-batch consistency documentation by CE (Capillary Electrophoresis) on seven charges of SCTL manufactured under GMP restrictions**

ferent batches of the combinatorial tetrapeptide library was demonstrated as shown in figure 3.

Assay for the biological standardisation of GMP-manufactured SCTL batches

Mast cells contain the serine proteases tryptase and trypsin. Keratinocytes of human skin dispose receptors which are activated by serine proteases leading to changes in cell function which are not yet fully recognized and understood. In our search for an *in-vitro* assay suitable for the biochemically standardized manufacture of SCTL batches, we have realized that SCTL inhibits tryptase and trypsin (Fig. 6). This way, we established this inhibitory action of SCTL on the enzymes as a quality control assay for the biochemical standardization of GMP-manufactured SCTL batches [Birr, 2005] .

General Preclinical Considerations on SCTL applications

HTX as a partially hydrolyzed extract of calf thymus containing a mixture of short tissue-specific peptides and SCTL as its synthetic correlate might be compared to thymus peptides regarding their pharmacodynamics.

A number of crude thymus extracts and subsequently purified peptides with distinct biological properties have been prepared from thymus tissue and blood. The most important preparations are summarized in Table 1 [Schulof, 1985, Cazzola et al., 1987].

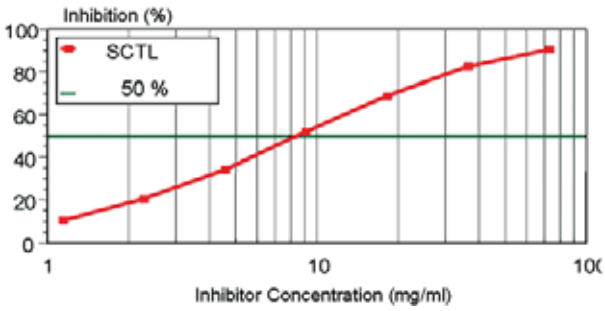


Fig. 4. **Inhibitory Activity of SCTL on Tryptase**

Action of Thymic Factors on T-cells

All of the naturally occurring thymus peptides mentioned above have been shown to augment T cell number and to modulate various T cell functions in man. Although many of the known thymic factors have similar activities in certain biological assays, it is likely that the respective thymic factors act essentially at different steps of the T-cell maturation [Cunningham-Rundles et al., 1999]. In general, studies utilizing peripheral blood lymphocytes were aimed at assessing the influence of thymic factors on either T cell number or on T cell function, as well as on the maturation and differentiation of pre-T lymphocytes [Birr, 1993, 1994, 1996; Ciardelli et al., 1982].

Table 4. **Thymic Factors isolated from thymus tissue and characterized**

Name	Abbr.	Content, size	Status	Origin
Thymosin		Mixture of polypeptides	Crude	Rat thymus
Thymic factor X	TFX	Mixture of polypeptides, MW rang-ing: 2,000-18,000 D	Crude	Calf thymus
Thymosin fraction 5	TF5	10 Major and 30 minor polypeptides	Purified	Calf thymus
Thymostimulin	TP-1	Mixture of polypeptides, MW rang-ing: 1,000-12,000 D	Purified	Calf thymus
Thymomodulin		Mixture of peptides	Purified	Calf thymus
Prothymosin-α α,	Ta	113 amino acids	Purified	Rat thymus
Thymopoietin	TP	Polypeptide, 5,562 D	Purified	
Thymulin, Facteur Thymique Serique	FTS- Zn	Nonapeptide, 847 D	Purified	Pig serum

Abbr.: abbreviations; D: Daltons.

Effects of SCTL on Peripheral Blood Lymphocytes

The T cell activating property of SCTL was compared to that of HTX in two independent studies after prestimulation by phythemagglutinin (PHA; 0.05–0.4 μg/ml).

In one study performed with blood from only 2 donors, SCTL (25–100 μg/ml) increased the proliferation rate (e.g. by approx. 110% at 100 μg/ml), whereas HTX (25–100 μg/ml) caused a dose-dependent inhibition (e.g. by approx. 53% at 100 μg/ml) [Hirt, ORPEGEN 1996; Ho et al., 1987].

IL-2 is released by activated T- cells and plays a pivotal role in sustaining both proliferative and cytotoxic immune responses. INF-γ augments T-cell cytotoxic activity but exhibits antiproliferative effects.

The effect of SCTL and HTX on PHA-induced and basal IL-2 secretion was investigated in human blood samples [Maurer, 1999].

In the presence of PHA (2.0 μg/ml), HTX (10–500 μg/ml) was ineffective. Only at the highest concentration used, SCTL (10–500 μg/ml) diminished IL-2 secretion, though without statistical significance ($P = 0.09$). In the absence of PHA, no statistically significant effects could be observed with either compound. However, the present data may suggest that by increasing the number of determinations/group (only 3 blood samples/group were used in the present study) a stimulation of IL-2 secretion by each compound might become statistically significant.

Substance	Dose (μg/ml)	Effect	Significance
Control	0	1	$P \leq 0.01$
PHA	0.4	2.33	
SCTL	10	1.38	$P = 0.03$
SCTL	50	1.46	
SCTL	100	1.82	
SCTL	200	1.49	
SCTL	500	1.21	

In the other study with blood of four donors, SCTL did not affect lymphocyte proliferation up to a dose of 500 μg/ml [Maurer, 1999]. HTX (10–500 μg/ml) exhibited the tendency to inhibit cell proliferation in a dose-dependent manner, but this effect was not statistically significant at the 5% level even at the highest concentration. When the effects of HTX and SCTL on lymphocyte proliferation were investigated in the absence of PHA, SCTL significantly stimulated cell proliferation at a single concentration, namely at 100 pg/ml ($P = 0.03$), whereas HTX had no effect. Summarizing these preliminary data, it may be suggested that SCTL and HTX differentially affect lymphocyte proliferation: Some concentrations of SCTL may cause an increase of basal proliferation which is not affected by HTX, whereas stimulated proliferation is virtually not changed by SCTL, but may be inhibited by HTX. However, considering the limited number of individual donors included in these studies a final conclusion cannot be drawn from the present data.

Thymic factors increase the synthesis of soluble mediators by T-cells, most notably, T-cell growth factor (TCGF or IL-2) and gamma Interferon (INF-γ). This cytokine reactivity pattern is defined as T-helper type 1 response.

In another series of experiments, the effect of SCTL and HTX on IL-2-induced cytotoxicity was compared with each other [Maurer, 1999]. Leukocyte YT-cells with properties similar to natural killer (NK) cells were used in this study. YT-cells were co-incubated with K 562 target cells (20:1) preloaded with calcein AM fluorescence dye. Calcein release was used as a measure of cytotoxicity. SCTL (10, 50, and 250 μg/ml) decreased the cytotoxicity induced by IL-2 at each concentration investigated ($P < 0.01$), whereas HTX (10, 50, and 250 μg/ml) reduced cytotoxicity only at the highest concentration used ($P < 0.05$). In summary, both SCTL and HTX may inhibit IL-2 mediated cytotoxicity, the latter, however, with much lower potency.

In conclusion, thymic factors, HTX and SCTL increase the number of peripheral blood lymphocytes, activate mature and precursor cells and increase the production of IL-2 and

INF-γ via T-helper type 1 response mechanism. Possibly, thymic factors, HTX and SCTL may be capable of exerting a homeostatic role in diseases associated with an imbalance of immunoregulatory T-cell activity. It seems that, for example, well defined thymic peptides like Thymosin-α_1 [Birr et al., 1979] may be required for an early step of cortical thymocyte maturation, whereas the other defined peptide, TP-5 appears to be involved in later stages.

Local Tolerance

Examining SCTL for acute skin irritation in rabbits the fur was removed by shaving from the dorsal area of the trunk of the animals approximately 24 h before the test. Care was taken to avoid abrading the skin; only animals with intact skin were used.

A dose of 500 mg was applied on the test side and then covered with a gauze patch, which was fixed with non-irritating tape for 4 h. The surrounding untreated skin served as a control. The skin sites were evaluated before the application of the test substance. After the exposure period the patch was removed and the skin was evaluated. Scores were taken 60 min as well as 14, 48 and 72 hours after removal. Under the present conditions none of the 3 rabbits exposed to 500mg SCTL showed substance related lesions. There were also no systemic intolerance reactions observed [Leuschner, 1997].

Moreover, SCTL and HTX were also tested in the EpiDerm®Skin Model (MatTek Corporation, Ashland, USA) for dermal irritation. This model consists of several layers of human keratinocytes and mimics human skin. The model substitutes for animal models used for testing skin irritation. In this model HTX and SCTL in a concentration of 10% in aqueous solution were not skin irritating [NeuroBiotec, 2005].

Toxicity

Acute toxicity of SCTL was investigated after a single i.v. injection to rats [Leuschner, 1997]. A dose of 10mg/kg for the rat was used in the experiment. The appropriate solution was administered once i.v. at the above mentioned dose to 1 group of 10 animals. Subsequently the animals were observed at 5, 15, 30 and 60 minutes as well at 6 and 24 hours after the administration. After a 14 day observation period the animals were autopsied. No animal died, and in none of them any substance related findings were observed. Also at autopsy no findings were noted. The LD 50 could not be calculated yet, because not lethality had occurred in the rats. Repeated dose toxicity studies have not been performed so far for SCTL.

In summary, the study reveals a very low if any acute intravenous toxicity of SCTL. Deducted from this it is very unlikely that topical application of SCTL in lotions or cremes can cause toxicity.

The mutagenic potential of SCTL was examined in Salmonella typhimurium strains TA 98, TA 102, TA1535 and TA 1537, without and with metabolic activation by Aroclor. In the first experiment after treatment with 10000 µg SCTL/plate without metabolic activation complete cytotoxicity was observed for tester strain TA 102.

In the second experiment with metabolic activation complete cytotoxicity was observed for tester strains TA 98, TA 102 and TA 1537. A marginal toxicity was observed at the same dose for TA 1535 in both experiments and for the strains TA 98 and TA 1537 in the first experiment. A marginal cytotoxicity had been observed at 3160 µg/plate for the tester strain TA 1537.

In these experiments no mutagenic effect was observed for SCTL tested up to cytotoxic concentrations (3160 and 10000 µg/plate) in any of the 5 tester strains in two independent experiments with and without metabolic activation [Leuschner, 1997].

Preliminary Considerations on Clinical SCTL Applications

The GMP-based standardized manufacture of the drug substance SCTL has been established. The Common Technical Document on SCTL has been compiled by ORPEGEN and presented at the FDA and the German BfARM for an IND approval on applications in clinical trials of different hair loss etiologies by the sponsor.

The author is aiming at clinical applications of SCTL in repairing the destructions of the cellular immune response resulting from virostatic and cytostatic chemo- and X-ray therapies. To date positive results are available only from private treatment of patients having suffered from opportunistic infections after chemotherapy. SCTL will be studied in topic, oral and parenteral applications probably at the NCT Heidelberg.

In another study towards prevention or reduction of alleric responses, the application of SCTL as a dietary supplement is considered. However, sponsors for these clinical applications of SCTL have not yet been identified.

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General remark: In other publ. GKL-02 stands for SCTL, and GKL-01 for HTX, respectively.

РОЗРОБЛЕННЯ ЛІКАРСЬКОЇ СУБСТАНЦІЇ НА ОСНОВІ ГМФ (SCTL) ДЛЯ ПРОФІЛАКТИКИ ОПОРТУНІСТИЧНИХ ІНФЕКЦІЙ ПІСЛЯ РЕНТГЕНІВСЬКОГО ОПРОМІНЕННЯ ТА ХІМІОТЕРАПІЇ РАКУ. ЗАМІНА БІБЛІОТЕКИ СИНТЕТИЧНОГО КОМБІНАТОРНОГО ТЕТРАПЕПТИДУ НА ЕКСТРАКТ ТИМУСА ТЕЛЯТИ

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SCTL є повністю синтетичним корелятом частково ензиматично гідролізованого екстракту тимуса теляти. Аби повністю виключити передачу бичачого пріонного енцефаліту за введення екстрактів тимуса, було створено продукт, який є повністю синтетичним корелятом активних компонентів гідролізату тканини тимуса, — SCTL. Бібліотека цього синтетичного пептиду повністю замінила компоненти екстракту тканини тимуса теляти в декількох косметичних та фармацевтичних препаратах. Активні компоненти SCTL були винаходом автора, однак застосування лікарської субстанції на його основі в косметичній та фармацевтичній промисловості здійснено іншими. Для SCTL описано лише обмежену кількість фармакологічних і токсикологічних властивостей. Було з'ясовано деякі важливі аспекти біологічної активності SCTL, які могли б до певної міри пояснити механізми його дії та клінічної ефективності.

Ключові слова: синтетичний корелят частково ензиматично гидролізованого екстракту тимуса теляти.

РАЗРАБОТКА ЛЕКАРСТВЕННОЙ СУБСТАНЦИИ НА ОСНОВЕ ГМФ (SCTL) ДЛЯ ПРОФИЛАКТИКИ ОППОРТУНИСТИЧЕСКИХ ИНФЕКЦИЙ ПОСЛЕ РЕНТГЕНОВСКОГО ОБЛУЧЕНИЯ И ХИМИОТЕРАПИИ РАКА. ЗАМЕНА БИБЛИОТЕКИ СИНТЕТИЧЕСКОГО КОМБИНАТОРНОГО ТЕТРАПЕПТИДА НА ЭКСТРАКТ ТИМУСА ТЕЛЕНКА

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SCTL является полностью синтетическим коррелятом частично энзиматически гидролизованного экстракта тимуса теленка. Чтобы полностью исключить передачу бычьего прионного энцефалита при введении экстрактов тимуса, был создан продукт, который является полностью синтетическим коррелятом активных компонентов гидролизата ткани тимуса, — SCTL. Библиотека этого синтетического пептида полностью заменила компоненты экстракта ткани тимуса теленка в нескольких косметических и фармацевтических препаратах. Активные компоненты SCTL были изобретением автора, однако применение лекарственной субстанции на его основе в косметической и фармацевтической промышленности осуществлено другими. Для SCTL описано лишь ограниченное количество фармакологических и токсикологических свойств. Были выяснены некоторые важные аспекты биологической активности SCTL, которые могли бы до некоторой степени объяснить механизмы его действия и клинической эффективности.

Ключевые слова: синтетический коррелят частично энзиматически гидролизованного экстракта тимуса теленка.

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THE VITAL IMPORTANCE OF PROVIDING SOUND SCIENTIFIC ADVICE TO POLICY MAKERS IN GOVERNMENT

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The article gives an idea of the scope of professional activity of scientists working in the field of biosafety in terms of providing timely and effective advice for politicians and diplomats in the government. It should be acknowledged that politicians and diplomats are also involved in a varying degree with biosafety issues such as toxicological and biological weapons, formulated in the relevant Convention: *Biological and Toxin Weapons Convention*. However taking into account their professional interests, they mightn't have appropriate information on relevant events in these and other activities. The value of these activities of qualified scientists knowing the latest information in the field of biosafety is difficult to overestimate, as they have the possibility to analyze any situation on the range of relevant activities and use their knowledge to make informed proposals which could be acceptable for their co-worker scientists in other areas of biological science. For highly qualified scientists such activities appeared to be effective, it is a vital aspect of their professional activity, because such scientists are able to provide scientific advice, analyze and summarize relevant scientific aspects on a specific topic of interest for politicians and diplomats. Such an analysis should include identification of key elements that are relevant to a given scientific problem and should be formulated so as the consequences of the various elements of the Convention were clearly appreciated and understood by politicians and diplomats. In other words, the relevant scientific aspects should be analyzed, summarized and presented in the context of the Convention, together with suggestions on what steps in this direction should be taken by politicians and diplomats.

Key words: Convention on biological and toxicological arms, recommendations of scientists, biosafety.

1. The Biological and Toxin Weapons Convention (BTWC) was opened for signature on 10 April 1972 and entered into force on 26 March 1975. The Co-Depositaries for this Convention are the Russian Federation, the United Kingdom and the United States. This Convention was the first one to totally prohibit the development, production and stockpiling of a class of weapons of mass destruction — that involving the use of biological agents and toxins as weapons. The Ukraine was one of the original States Parties who signed the Convention on 10 April 1972 and for whom the Convention entered into force on 26 March 1975. The central prohibition is set out in Article I of the Convention [1] under which:

Each State Party to this Convention undertakes never in any circumstances to develop, produce, stockpile or otherwise acquire or retain:

(1) Microbial or other biological agents, or toxins whatever their origin or method of production, of types and quantities that have no justification for prophylactic, protective or other peaceful purposes;

(2) Weapons, equipment or means of delivery designed to use such agents or toxins for hostile purposes or in armed conflict.

2. In accordance with Article XII of the Convention, Review Conferences have been held at five year intervals — in 1981, 1986, 1991, 1996, 2001/2, 2006 and 2011 — *to*

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review the operation of the Convention, with a view to ensuring that the purposes of the preamble of the Convention, ... are being realized. In addition, it is required that *Such review shall take into account any new scientific and technological developments relevant to the Convention.* It is thus evident that the continuing and effective implementation of the Convention requires that the States Parties carrying out the five yearly Review Conferences need to be advised of any relevant new scientific and technological developments and of what their significance is for the Convention and what should be done in order to ensure that the effectiveness of the Convention is maintained.

3. The need to be able to provide sound scientific advice to policy makers and diplomats in government in each State Party is thus enshrined in the BTWC. And, it is also evident that the delegations sent by States Parties to the Review Conferences will need to include qualified and knowledgeable scientists who will be able to provide advice to the delegations during the Review Conferences on approaches to be adopted by the delegation as well as how to respond to scientific and technical information submitted and recommendations made by other delegations.

4. Although Review Conferences take place at five year intervals, the practice has been adopted since the decision of the Fifth Review Conference in 2002, to have an intersessional programme during which the States Parties are each year *to discuss and promote common understandings and effective action* on specified topics first at an annual Meeting of Experts and then subsequently at an annual Meeting of States Parties. As might be expected, the delegations sent to the Meeting of Experts need to include qualified and knowledgeable scientists who will be able to provide advice to the delegations during the Meeting of Experts at which scientific and technical details are discussed and debated in regard to the specific items so as to ensure that the ideas captured in the Annex to the Meeting of Experts are correctly expressed and can lead to being agreed in the substantive paragraphs of the subsequent Meeting of States Parties later that year that will be considering the same specific topics.

QUALIFIED AND KNOWLEDGEABLE SCIENTISTS

5. For scientists to be effective in providing advice to the policy makers and the diplomats, there are several essential characteristics that the scientist is required to have.

Awareness and understanding of the BTWC

6. An essential prerequisite for any scientist providing advice to policy makers and diplomats on the BTWC is an understanding of the Convention and an appreciation of what the various Articles of the Convention oblige States Parties to do. In addition, the scientist needs to be aware of the extended understandings that have been agreed by the States Parties at the successive Review Conferences. This overall awareness and understanding is essential if the advice is going to be credible and helpful to the policy makers and diplomats.

7. In addition, the advice will be much more effective if the scientist is also aware of what the last Review Conference has decided — the Final Report of the Review Conference and, in particular, its *Final Declaration* and its *Decisions and Recommendations* set the scene for the current Intersessional Period and have decided the specific topics to be discussed in order to *promote common understandings and effective action.*

Ability to analyse, summarise and express relevant scientific aspects

8. Another essential prerequisite is that the scientist providing scientific advice is able to analyse and summarise the relevant scientific aspects of the particular topic on which advice is to be given to the policy makers and diplomats. Such analysis has to be able to identify the key elements that are relevant and these need to be expressed so that the implications in regard to the various elements of the Convention are clearly appreciated and understood by the policy makers and diplomats. In other words, the relevant scientific aspects have to be analysed, summarized and presented in the **context** of the Convention together with proposals as to what steps should be taken by the policy makers and diplomats. Throughout, the scientist has to be answering the question that the policy makers and diplomats will be asking themselves about the scientific aspects — «So what?» In other words, what do the scientific aspects mean in the context of the Convention.

Awareness of the risks to humans, animals and plants

9. One of the extended understandings that the States Parties have agreed at the successive Review Conferences is encapsulated in the language relating to Article I of the Convention in the Final Declaration [2] of the Seventh Review Conference that states:

1. The Conference reaffirms the importance of Article I, as it defines the scope of the Convention. The Conference declares that the

Convention is comprehensive in its scope and that all naturally or artificially created or altered microbial and other biological agents and toxins, as well as their components, regardless of their origin and method of production and whether they affect ***humans, animals or plants,*** *of types and in quantities that have no justification for prophylactic, protective or other peaceful purposes, are unequivocally covered by Article I.* [Emphasis added]

This makes it clear that the prohibition in the Convention applies to *microbial and other biological agents and toxins* that affect *humans, animals or plants.* It consequently follows that the scientists providing policy advice on the BTWC to the policy makers and diplomats need to be aware of the activities being carried out by the World Health Organization (WHO) [3], the World Animal Health Organization (OIE) [4] and the Food and Agriculture Organization (FAO) [5] in regard to countering outbreaks of human, animal and plant diseases and to promote human health security, animal health security and food security.

10. These scientists providing policy advice need also to be aware that the terminology used in regard to terms such as biosafety, biosecurity and food security can be different in the WHO, OIE and FAO arenas than that used in the context of the Convention.

Awareness of the risks to the environment

11. As the prohibition in the Convention relates to *microbial and other biological agents and toxins* that affect *humans, animals or plants*, it will be appreciated that there is a close relationship to the environment. Consequently, the scientists providing advice need also to be aware of the Conventions and Protocols that relate to the environment — and in particular to the Convention on Biological Diversity and its associated Cartagena Protocol on Biosafety.

The Convention on Biological Diversity (CBD) [6] was the result of an increasing global awareness that:

The Earth's biological resources are vital to humanity's economic and social development. As a result, there is a growing recognition that biological diversity is a global asset of tremendous value to present and future generations. At the same time, the threat to species and ecosystems has never been so great as it is today. Species extinction caused by human activities continues at an alarming rate.

The CBD was opened for signature on 5 June 1992 at the United Nations Conference on Environment and Development (the Rio «Earth Summit») and entered into force on 29 December 1993. It has currently 193 Parties to the Convention.

2. A particular element of the CBD arises from *Article 18 Technical and Scientific Cooperation* which requires that:

1. The Contracting Parties shall promote international technical and scientific cooperation in the field of conservation and sustainable use of biological diversity, where necessary, through the appropriate international and national institutions.

2. Each Contracting Party shall promote technical and scientific cooperation with other Contracting Parties, in particular developing countries, in implementing this Convention, inter alia, through the development and implementation of national policies. In promoting such cooperation, special attention should be given to the development and strengthening of national capabilities, by means of human resources development and institution building.

3. The Conference of the Parties, at its first meeting, shall determine how to establish a clearing-house mechanism to promote and facilitate technical and scientific cooperation.

The clearing-house mechanism addressed in paragraph 3 of Article 18 has as its mission the requirement to contribute significantly to the implementation of the Convention on Biological Diversity through effective information services and other appropriate means in order to promote and facilitate scientific and technical cooperation, knowledge sharing and information exchange, and to establish a fully operational network of Parties and partners.

13. Closely associated with the CBD is its Cartagena Protocol on Biosafety [7]. This addresses the movements of living modified organisms (LMOs) resulting from modern biotechnology from one country to another. It was adopted on 29 January 2000 as a supplementary agreement to the Convention on Biological Diversity and entered into force on 11 September 2003. It establishes an advance informed agreement (AIA) procedure for ensuring that countries are provided with the information necessary to make informed decisions before agreeing to the import of such organisms into their territory. The Protocol contains reference to a precautionary approach. The Protocol also establishes a Biosafety Clearing-House to facilitate the exchange of information on living modified organisms and to assist countries in the implementation of the Protocol. It has currently 166 Parties to the Protocol. The Ukraine Profile is available at http://bch.cbd.int/about/countryprofile.shtml?country=ua.

14. Article 22 of the Protocol addresses *Capacity Building* and requires that:

1. The Parties shall cooperate in the development and/or strengthening of human resources and institutional capacities in biosafety, including biotechnology to the extent that it is required for biosafety, for the purpose of the effective implementation of this Protocol, in developing country Parties, in particular the least developed and small island developing States among them, and in Parties with economies in transition, including through existing global, regional, subregional and national institutions and organizations and, as appropriate, through facilitating private sector involvement.

2. For the purposes of implementing paragraph 1 above, in relation to cooperation, the needs of developing country Parties, in particular the least developed and small island developing States among them, for financial resources and access to and transfer of technology and know-how in accordance with the relevant provisions of the Convention, shall be taken fully into account for capacity-building in biosafety. Cooperation in capacity-building shall, subject to the different situation, capabilities and requirements of each Party, include scientific and technical training in the proper and safe management of biotechnology, and in the use of risk assessment and risk management for biosafety, and the enhancement of technological and institutional capacities in biosafety. The needs of Parties with economies in transition shall also be taken fully into account for such capacity-building in biosafety.

15. Closely related to the biosafety requirements for the Protocol is the initiative taken by the United Nations Environment Programme to implement the *Global Environment Facility (GEF) Initial Strategy on Biosafety* [8] which was adopted in November 2000. This Strategy aims to assist countries preparing for the Cartagena Biosafety Protocol through the establishment of National Biosafety Frameworks (NBFs). The NBF is a combination of policy, legal, administrative and technical instruments that are set in place to address safety for the environment and human health in relation to modern biotechnology. As at 30th May 2012, 119 countries have completed the majority of development of their National Biosafety Projects and their draft NBFs are available online [9]. This includes the draft National Biosafety Framework for the Ukraine. These projects have generated a wealth of in-country experience in building capacity for biosafety.

16. These National Biosafety Frameworks are highly relevant to the initiatives being taken to strengthen biosafety and biosecurity under the BTWC and scientific experts advising national diplomats and policy makers need to be aware of their relevance.

17. There are also, as might be expected, relevant activities in regard to education and awareness. Thus the Convention on Biological Diversity in its Article 13 *Public Education and Awareness* requires that:

The Contracting Parties shall:

(a) Promote and encourage understanding of the importance of, and the measures required for, the conservation of biological diversity, as well as its propagation through media, and the inclusion of these topics in educational programmes; and

(b) Cooperate, as appropriate, with other States and international organizations in developing educational and public awareness programmes, with respect to conservation and sustainable use of biological diversity.

Likewise the Cartagena Protocol on Biosafety in its Article 23 *Public Awareness and Education* requires that:

1. The Parties shall:

(a) Promote and facilitate public awareness, education and participation concerning the safe transfer, handling and use of living modified organisms in relation to the conservation and sustainable use of biological diversity, taking also into account risks to human health. In doing so, the Parties shall cooperate, as appropriate, with other States and international bodies;

(b) Endeavour to ensure that public awareness and education encompass access to information on living modified organisms identified in accordance with this Protocol that may be imported.

There are consequently clear benefits to States Parties to the BTWC if those scientists providing advice to diplomats and policy makers are aware of the international and national activities associated with the Convention on Biological Diversity and its Cartagena Protocol on Biosafety.

Awareness of CBRN risks and preparedness

18. Whilst it may be thought that scientists providing advice to diplomats and policy makers need only be aware of the life sciences, their advice will be much more effective if they are also aware of the broader chemical, biological, radiological and nuclear risks as to an increasing extent countries are coordinating their preparedness for CBRN incidents whether accidental or deliberate.

19. **The Chemical Weapons Convention.** The CWC [10] is closely related to the BTWC and it prohibits the development, production and stockpiling of chemical weapons. In Article I *General Obligations* it requires that:

1. Each State Party to this Convention undertakes never under any circumstances:

(a) To develop, produce, otherwise acquire, stockpile or retain chemical weapons, or transfer, directly or indirectly, chemical weapons to anyone;

(b) To use chemical weapons;

(c) To engage in any military preparations to use chemical weapons;

(d) To assist, encourage or induce, in any way, anyone to engage in any activity prohibited to a State Party under this Convention.

and in its Article II *Definitions and Criteria* states that:

For the purposes of this Convention:

1. «Chemical Weapons» means the following, together or separately:

(a) Toxic chemicals and their precursors, except where intended for purposes not prohibited under this Convention, as long as the types and quantities are consistent with such purposes;

(b) Munitions and devices, specifically designed to cause death or other harm through the toxic properties of those toxic chemicals specified in subparagraph (a), which would be released as a result of the employment of such munitions and devices;

(c) Any equipment specifically designed for use directly in connection with the employment of munitions and devices specified in subparagraph (b).

2. «Toxic Chemical» means:

Any chemical which through its chemical action on life processes can cause death, temporary incapacitation or permanent harm to humans or animals. This includes all such chemicals, regardless of their origin or of their method of production, and regardless of whether they are produced in facilities, in munitions or elsewhere.

As the BTWC addresses *Microbial or other biological agents, or toxins whatever their origin or method of production* it is evident that there is — and rightly so — an overlap between the two Conventions with chemicals such as toxins being prohibited under both Conventions.

20. The Chemical Weapons Convention opened for signature on 13 January 1993 and entered into force on 29 April 1997. It currently has 188 States Parties. The CWC has concentrated in its first fifteen years on the destruction of declared chemical weapons stockpiles — the Convention in Article IV requires that *Each State Party shall destroy all chemical weapons ... Such destruction ... shall finish no later than 10 years after entry into force of the Convention.* In addition, Part IV(A) of the Verification Annex to the Convention includes provisions should a State Party believe that *it will be unable to ensure the destruction of all Category 1 chemical weapons not later than 10 years after the entry into force of this Convention* then the Executive Council can grant an extension — however, it is specified that *Any extension shall be the minimum necessary, but in no case shall the deadline for a State Party to complete its destruction of all chemical weapons be extended beyond 15 years after the entry into force of this Convention.* The Convention is now focusing much more on the non-proliferation and other requirements required by the Convention and the activities of the Organisation for the Prohibition of Chemical Weapons are more closely related to those being carried out by the States Parties to the BTWC.

21. **The 1925 Geneva Protocol.** This is the *Protocol for the Prohibition of the Use in War of Asphyxiating, Poisonous or other Gases, and of Bacteriological Methods of Warfare* [11] which was signed at Geneva on 17 June 1925. This notes that:

Whereas the use in war of asphyxiating, poisonous or other gases, and of all analogous liquids, materials or devices, has been justly condemned by the general opinion of the civilised world; and

Whereas the prohibition of such use has been declared in Treaties to which the majority of Powers of the world are Parties; and

To the end that this prohibition shall be universally accepted as a part of International

Law, binding alike the conscience and the practice of nations;

and goes on to declare that:

Declare:

That the High Contracting Parties, so far as they are not already Parties to Treaties prohibiting such use, accept this prohibition, agree to extend this prohibition to the use of bacteriological methods of warfare and agree to be bound as between themselves according to the terms of this declaration.

The High Contracting Parties will exert every effort to induce other States to accede to the present Protocol. Such accession will be notified to the Government of the French Republic, and by the latter to all signatory and

acceding Powers, and will take effect on the date of the notification by the Government of the French Republic.

22. It should be noted that at the Seventh Review Conference of the BTWC, the States Parties agreed in their Final Declaration [12] in regard to Article VIII of the BTWC that:

41. The Conference appeals to all States Parties to the 1925 Geneva Protocol to fulfil

their obligations assumed under that Protocol and urges all states not yet party to the Protocol to ratify or accede to it without further delay.

42. The Conference acknowledges that the 1925 Geneva Protocol, which prohibits the use in war of asphyxiating, poisonous or other gases, and of bacteriological methods of warfare, and the Convention complement each other. The Conference reaffirms that nothing contained in the Convention shall be interpreted as in any way limiting or detracting from the obligations assumed by any state under the 1925 Geneva Protocol.

43. The Conference stresses the importance of the withdrawal of all reservations to the 1925 Geneva Protocol related to the Convention.

44. The Conference recalls the actions which States Parties have taken to withdraw their reservations to the 1925 Geneva Protocol related to the Convention, and calls upon those States Parties that continue to maintain pertinent reservations to the 1925 Geneva Protocol to withdraw those reservations, and to notify the Depositary of the 1925 Geneva Protocol accordingly, without delay.

45. The Conference notes that reservations concerning retaliation, through the use of any of the objects prohibited by the Convention, even conditional, are totally incompatible with the absolute and universal prohibition of the development, production, stockpiling, acquisition and retention of bacteriological (biological) and toxin weapons, with the aim to exclude completely and forever the possibility of their use.

46. The Conference notes that the Secretary-General's investigation mechanism, set out in A/44/561 and endorsed by the General Assembly in its resolution 45/57, represents an international institutional mechanism for investigating cases of alleged use of biological or toxin weapons. The Conference notes national initiatives to provide relevant training to experts that could support the Secretary-General's investigative mechanism.

23. Universal adherence to the Geneva Protocol by all States, including all States Parties to the BTWC, has been an agreed politically binding commitment since 1980, reaffirmed by every subsequent Review Conference that has issued a *Final Declaration*. It should be noted that the original statement, in the *Final Declaration* which the First Review Conference agreed on 21 March 1980, called on all States not yet parties to the Geneva Protocol to ratify or accede to it at the earliest possible date; the Second Review Conference on 26 September 1986 urged them to adhere to it at the earliest possible date; the Third Review Conference on 27 September 1991 urged them to accede to it without delay, as did the Fourth Review Conference on 6 December 1996, the Sixth Review Conference on 8 December 2006 and the Seventh Review Conference on 22 December 2011.

24. **Secretary-General Mechanism for Investigation of Alleged Use** [13]. Article VI of the BTWC states that:

(1) Any State Party to this Convention which finds that any other State Party is acting in breach of obligations deriving from the provisions of the Convention may lodge a complaint with the Security Council of the United Nations. Such a complaint should include all possible evidence confirming its validity, as well as a request for its consideration by the Security Council.

(2) Each State Party to this Convention undertakes to cooperate in carrying out any investigation which the Security Council may initiate, in accordance with the provisions of the Charter of the United Nations, on the basis of the complaint received by the Council. The Security Council shall inform the States Parties to the Convention of the results of the investigation.

25. At the Seventh Review Conference, the States Parties to the BTWC agreed in their Final Declaration [14] in regard to Article VI that:

29. The Conference invites the Security Council:

(a) to consider immediately any complaint lodged under this Article and to initiate any measures it considers necessary for the investigation of the complaint in accordance with the Charter;

(b) to request, if it deems necessary and in accordance with its Resolution 620 of 1988, the United Nations Secretary-General to investigate the allegation of use, using the technical guidelines and procedures contained in Annex I of United Nations Document A/44/561;

(c) to inform each State Party of the results of any investigation initiated under this Article and to consider promptly any appropriate further action which may be necessary.

26. The Secretary-General's Mechanism (SGM) for the investigation of alleged use of chemical, biological and toxin weapons derives from a mandate established by the United Nations General Assembly in its resolution 42/37C of 1987 and reaffirmed by the United Nations Security Council through its resolution 620 (1988). Under this mandate, the Secretary-General is requested *to carry out investigations in response to reports that may be brought to his attention by any Member State concerning the possible use of chemical and bacteriological (biological) or toxin weapons that may constitute a violation of the Geneva Protocol or other relevant rules of customary international law in order to ascertain the facts of the matter and to report promptly the results of any such investigations to all Member States.*

Investigations of alleged use will be conducted according to technical guidelines and procedures (A/44/561 Annex I) which were endorsed by the General Assembly in 1990. The appendices to the technical guidelines and procedures were updated in 2007.

27. **Security Council Resolution 1540(2004) [15].** In 2004, the Security Council decided that all States shall refrain from providing any form of support to non-State actors who attempt to develop, acquire, manufacture, possess, transport, transfer or use nuclear, chemical or biological weapons and their means of delivery, and requires all States to adopt and enforce appropriate effective laws to this effect. The resolution also requires all States to establish various types of domestic controls to prevent the proliferation of such weapons and their related materials. A Security Council Committee was established pursuant to resolution 1540 to report to the Council on the implementation of the resolution. Security Council resolution 1977 (2011) extended the mandate of the Committee until 25 April 2021.

28. The 1540 Committee has prepared matrices for 179 of the Member States of the United Nations which following approval by the Committee in November and December 2010 are available at http://www.un.org/sc/1540/1540matrix.shtml. This in *OP 2 — Biological Weapons (BW)* requests States to answer the question:

Does national legislation exist which prohibits persons or entities to engage in one of the following activities? Can violators be penalized?

in regard to some 14 activities:

1. *Manufacture/produce*
2. *Acquire*
3. *Possess*
4. *Stockpile/store*
5. *Develop*
6. *Transport*
7. *Transfer*
8. *Use*
9. *Participate as an accomplice in above-mentioned activities*
10. *Assist in above-mentioned activities*
11. *Finance above-mentioned activities*
12. *Above-mentioned. activities related to means of delivery*
13. *Involvement of non-State actors in above-mentioned. activities*
14. *Other*

29. In addition, the matrix in *OP 3 (a) and (b) — Account for/Secure/Physically protect BW including Related Materials* requests States to answer the question:

Are any of the following measures, procedures or legislation in place to account for, secure or otherwise protect BW and Related Materials? Can violators be penalized ?

in regard to some 17 categories:

1. *Measures to account for production*
2. *Measures to account for use*
3. *Measures to account for storage*
4. *Measures to account for transport*
5. *Other measures for accounting*
6. *Measures to secure production*
7. *Measures to secure use*
8. *Measures to secure storage*
9. *Measures to secure transport*
10. *Other measures for securing*
11. *Regulations for physical protection of facilities/materials/ transports*
12. *Licensing/registration of facilities/persons handling biological materials*
13. *Reliability check of personnel*
14. *Measures to account for/secure/ physically protect means of delivery*
15. *Regulations for genetic engineering work*
16. *Other legislation/ regulations related to safety and security of biological materials*
17. *Other.*

30. The matrix in *OP 3 (c) and (d) and related matters from OP 6 and OP 10 — Controls of BW including Related Materials* requests States to answer the question:

Which of the following legislation, procedures, measures, agencies exist to control border crossings, export/import and other transfers of BW and Related Materials? Can violators be penalized?

in regard to some 26 categories:

1. *Border control*
2. *Technical support of border control measures*

3. *Control of brokering, trading in, negotiating, otherwise assisting in sale of goods and technology*
4. *Enforcement agencies/authorities*
5. *Export control legislation in place*
6. *Licensing provisions*
7. *Individual licensing*
8. *General licensing*
9. *Exceptions from licensing*
10. *Licensing of deemed export/visa*
11. *National licensing authority*
12. *Interagency review for licenses*
13. *Control lists*
14. *Updating of lists*
15. *Inclusion of technologies*
16. *Inclusion of means of delivery*
17. *End-user controls*
18. *Catch-all clause*
19. *Intangible transfers*
20. *Transit control*
21. *Trans-shipment control*
22. *Re-export control*
23. *Control of providing funds*
24. *Control of providing transport services*
25. *Control of importation*
26. *Extraterritorial applicability*
27. *Other.*

These matrices provide a valuable resource that can be drawn upon by the States Parties to the BTWC in promoting the implementation of Article IV of the Convention.

31. The completed matrices for the Ukraine are available at:

http://www.un.org/en/sc/1540/docs/matrices/Ukraine revised matrix.pdf

and provide detailed information on the legislation and procedures that apply in the Ukraine in regard to activities relating to biological weapons as well as to chemical and nuclear weapons.

32. **European Union Public Health Preparedness for Cross-Border Health Threats [16].** On 8 December 2011 the European Commission adopted a legislative proposal on the means to address serious cross-border health threats. This proposal is to extend the existing co-ordination mechanism for communicable diseases to all heath threats caused by biological, chemical or environmental causes. It provides for the assessment of risks and the co-ordination of measures from communicable diseases to be extended to all heath threats caused by biological, chemical or environmental causes.

33. **United Nations Interregional Crime and Justice Research Institute (UNICRI) CBRN Risk Mitigation and Security Governance Programme** [17]. This was launched in 2004, in conjunction with the IAEA, the OPCW, the ISU of the BTWC, the WHO, INTERPOL and the World Customs Organization (WCO, to support the development of an **integrated CBRN approach** that incorporates all international, regional and national CBRN components into a common strategy. This entails the application of a **holistic approach** through which all stakeholders, while operating autonomously, can establish common goals, identify and manage resources to achieve these goals, clearly allocate responsibilities and tasks, elaborate functioning channels of communication, create a security culture based on common learning, and ensure that lessons learnt are incorporated and absorbed throughout the whole system.

34. A closely associated element of this programme is to establish regional **CBRN Centres of Excellence** [18]. Such centres are seen as a cornerstone of these activities by offering a coherent and comprehensive approach covering legal, regulatory, enforcement and technical issues. It is evident that a number of projects will involve the Ukraine including one launched in January 2013 entitled *Knowledge development and transfer of best practice on bio-safety, bio-security, bio-risk management*.

Conclusions

35. This paper has sought to give an indication of the breadth of activities that scientists need to be aware of if they are to be effective in providing advice to the policy makers and the diplomats in Government. It needs to be recognised that the policy makers and diplomats engaged in a particular activity such as the Biological and Toxin Weapons Convention (BTWC) may well not be involved in some of the other relevant activities and thus be unaware of relevant developments in these other activities. The value that qualified and knowledgeable scientists can bring is thus considerable as they are able to analyse the situation across the range of relevant activities and can use their knowledge to make soundly based proposals that will frequently be acceptable to their fellow scientists in other delegations.

36. For the qualified and knowledgeable scientists to be effective it is vital that the scientist providing scientific advice is able to analyse and summarise the relevant scientific aspects of the particular topic on which advice is to be given to the policy makers and diplomats. Such analysis has to be able to identify the key elements that are relevant and these need to be expressed so that the implications in regard to the various elements of the Convention are clearly appreciated and understood by the policy makers and diplomats. In other words, the relevant scientific aspects have to be analysed, summarized and presented in the **context** of the Convention together with proposals as to what steps should be taken by the policy makers and diplomats.

37. These qualified and knowledgeable scientists also have a continuing responsibility once they have successfully explained the context of the relevant scientific aspects to the policy makers and diplomats to then follow through by encouraging the national policy makers to take appropriate action nationally to implement the changes that would enhance the effectiveness nationally of the implementation of the BTWC.

38. Qualified and knowledgeable scientists who are effective in providing advice to the policy makers and diplomats have an additional bonus — in that they are well qualified to assist in raising awareness and facilitating outreach to all those engaged in the life sciences about the issues and the progress being made by the States Parties in strengthening the effectiveness of the Biological and Toxin Weapons Convention and thereby helping to make it a safer world for all of us.

Postscript

39. I have chosen to write this article on *The Vital Importance of Providing Sound Scientific Advice to Policy Makers in Government* in recognition of the outstanding contributions that Ambassador Professor Dr. Serhiy Vasilyovich Komisarenko has made as a member of the delegation of the Ukraine, which he has frequently led, to the meetings of the States Parties to the Biological and Toxin Weapons Convention in Geneva over the past eight years.

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ВАЖЛИВІСТЬ І ЗНАЧУЩІСТЬ НАДАННЯ НАУКОВО ОБГРУНТОВАНИХ РЕКОМЕНДАЦІЙ ДЛЯ УРЯДОВЦІВ

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У статті подається уявлення про широту професійної діяльності вчених, які працюють у галузі біобезпеки, у плані надання своєчасних і ефективних рекомендацій для політиків і дипломатів в уряді. Політики та дипломати тією чи іншою мірою також займаються проблемами біобезпеки, такими, зокрема, як біологічна і токсикологічна зброя (сформульовані у відповідній конвенції: *Biological and Toxin Weapons Convention*), однак у них може бути відсутня відповідна інформація про певні події. З огляду на це значення діяльності кваліфікованих учених, що володіють останніми даними у сфері біобезпеки, важко переоцінити, адже вони можуть проаналізувати будь-яку ситуацію з усього спектра відповідної проблематики і використовувати свої знання для обґрунтованих пропозицій, які часто можуть бути прийнятними й для їхніх колег-вчених в інших галузях біологічної науки. Для висококваліфікованих учених подібна діяльність є ефективним і вкрай важливим аспектом їхньої професійної активності, оскільки вони в змозі надати наукові консультації, проаналізувати і узагальнити відповідні наукові дані з конкретної теми, що цікавить політиків і дипломатів. Такий аналіз передбачає ідентифікацію ключових елементів, які є актуальними для даної наукової проблеми і мають бути сформульовані таким чином, щоб наслідки оцінки різних елементів конвенції були чіткими та зрозумілими для політиків і дипломатів. Тобто, відповідні наукові аспекти мають бути проаналізовані, узагальнені та представлені в контексті конвенції разом із пропозиціями про те, які кроки в цьому напрямі слід зробити.

Ключові слова: Конвенція з біологічної і токсикологічної зброї, біобезпека.

ВАЖНОСТЬ И ЗНАЧИМОСТЬ ПРЕДОСТАВЛЕНИЯ НАУЧНО ОБОСНОВАННЫХ РЕКОМЕНДАЦИЙ ДЛЯ ЧЛЕНОВ ПРАВИТЕЛЬСТВА

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В статье дается представление о широте профессиональной деятельности ученых, работающих в сфере биобезопасности, в плане предоставления своевременных и эффективных рекомендаций для политиков и дипломатов в правительстве. Политики и дипломаты в той или иной степени также занимаются проблемами биобезопасности, такими, в частности, как биологическое и токсикологическое оружие (сформулированы в соответствующей конвенции: *Biological and Toxin Weapons Convention*), однако у них может отсутствовать соответствующая информация об определенных событиях. Учитывая это, значение деятельности квалифицированных ученых, располагающих последними данными в сфере биобезопасности, трудно переоценить, поскольку они могут проанализировать любую ситуацию по всему спектру соответствующей проблематики и использовать свои знания для внесения обоснованных предложений, которые часто могут быть приемлемы и для их коллег-ученых в других областях биологической науки. Для высококвалифицированных ученых подобная деятельность является эффективным и весьма важным аспектом их профессиональной активности, поскольку они могут предоставить научные консультации, проанализировать и обобщить соответствующие научные данные по конкретной теме, интересующей политиков и дипломатов. Подобный анализ должен включать идентификацию ключевых элементов, которые являются актуальными для данной научной проблемы и должны быть сформулированы таким образом, чтобы последствия оценки различных элементов конвенции были четкими и понятными для политиков и дипломатов. Следовательно, соответствующие научные аспекты должны быть проанализированы, обобщены и представлены в контексте конвенции вместе с предложениями о том, какие шаги в этом направлении следует предпринять.

Ключевые слова: Конвенция по биологическому и токсикологическому оружию, биобезопасность.

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ВІДПОВІДЬ ПУХЛИННИХ КЛІТИН НА ПРИГНІЧЕННЯ ЕКСПРЕСІЇ АДАПТЕРНОГО ПРОТЕЇНУ RUK/CIN85 РЕКОМБІНАНТНИМИ ЛЕНТИВІРУСАМИ

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Ruk/CIN85 є одним з адаптерних протеїнів, що відіграють важливу роль у регулюванні процесів проліферації, рухливості та загибелі клітин. Нещодавно нами було показано, що надекспресія Ruk/CIN85 посилює трансформувальний потенціал клітин аденокарциноми грудної залози людини лінії MCF-7. Метою цього дослідження було з'ясувати, чи впливатиме на біологічні властивості клітин пригнічення експресії Ruk/CIN85. Для РНК-інтерференції Ruk/CIN85 було одержано лентивірусні конструкції, що містять Ruk/CIN85-специфічні послідовності small hairpin RNA. За допомогою отриманих рекомбінантних лентивірусів встановлено, що пригнічення експресії Ruk/CIN85 впливає на біологічні властивості (рухливість, проліферацію, рівень експресії транспортера ABCG2, утворення активних форм кисню) різних типів пухлинних клітин, а саме: аденокарциноми грудної залози людини лінії MCF-7, аденокарциноми ободової кишки людини HT-29 та мишачої карциноми легень Льюїса.

Ключові слова: адаптерні протеїни, Ruk/CIN85, РНК-інтерференція, лентивірусні вектори, рухливість клітин, активні форми кисню.

Специфічність відповіді клітин на зовнішні стимули потребує інтеграції численних внутрішньоклітинних сигнальних шляхів. Однією з основних груп протеїнів, що відповідають за таку інтеграцію, є адаптерні та риштувальні (scaffold) протеїни, які забезпечують збирання надмолекулярних мультипротеїнових комплексів та їх регулювання [1]. Ці мультипротеїнові комплекси передають всередину клітини сигнали, залучені до контролю росту, диференціювання, адгезії, рухливості та виживання клітин. Адаптерний протеїн Ruk/CIN85 (Regulator for ubiquitous kinase/c-Cbl-interacting protein of 85 kDa) завдяки складній доменній організації та наявності множинних молекулярних форм бере участь у низці важливих внутрішньоклітинних сигнальних процесів, що лежать в основі регулювання архітектури актинового цитоскелета, адгезії, рухливості та інвазії клітин, апоптозу, мітогенного сигналювання, транспорту мембранних везикул, ліганд-опосередкованого ендоцитозу рецепторних тирозинових протеїнкіназ, інфікування клітин вірусом простого герпесу [2–5].

Нещодавно нами було показано, що надекспресія Ruk/CIN85 посилює трансформувальний потенціал клітин аденокарциноми грудної залози людини лінії MCF-7 [5]. Проте не було досліджено, який вплив на властивості клітин лінії MCF-7 з надекспресією Ruk/CIN85 матиме пригнічення експресії цього протеїну. Одним з найпоширеніших сучасних експериментальних підходів для дослідження функцій генів у культурах клітин є використання методології РНК-інтерференції [6]. Лентивірусні вектори, що кодують shRNA (small hairpin RNA), які вбудовуються в геном клітини-хазяїна, використовують для тривалого пригнічення (silencing) експресії відповідних генів [7]. З огляду на це, ми спрямували наші зусилля на конструювання рекомбінантних ленти-

вірусів, які містять послідовність shRNA для протеїну Ruk/CIN85. Одержання такої лентивірусної конструкції дало змогу пригнітити експресію Ruk/CIN85 у клітинах гліоми щура C6, аденокарциноми ободової кишки людини HT-29, у клітинах мишачої карциноми легень Льюїса (Lewis lung carcinoma, LLC) та аденокарциноми грудної залози людини лінії MCF-7 з надекспресією Ruk/CIN85, а також виявити, що пригнічення експресії Ruk/CIN85 може впливати на рухливість, проліферацію і утворення активних форм кисню (АФК) у зазначених типах клітин. Для того, щоб оцінити потенційний вплив надекспресії Ruk/CIN85 у клітинах MCF-7 на розвиток резистентності до протипухлинних препаратів, було також досліджено вміст одного з АТР-зв'язувальних мембранних касетних транспортерів ABCG2 (ATP-binding cassette sub-family G member 2), що відіграє роль у розвиткові раку грудної залози людини [8].

Матеріали і методи

Культура клітин. Клітини аденокарциноми грудної залози людини лінії MCF-7, ембріональної нирки людини HEK293T, аденокарциноми ободової кишки людини HT-29, мишачої карциноми LLC та гліоми щура C6 культивували в середовищі DMEM (PAA Laboratories, Австрія), яке містило 10% ембріональної сироватки теляти (fetal bovine serum, FBS), 50 U/мл пеніциліну та 100 мкг/мл стрептоміцину. Клітини утримували в CO_2-інкубаторі (Cytoperm 8080, Heraeus, Hanau, Німеччина) при 37 °C у зволоженій атмосфері, що містила 5% CO_2 (за об'ємом). Одержання сублінії клітин MCF-7 зі стабільною надекспресією протеїну Ruk/CIN85 (G4) описано раніше [5].

Одержання рекомбінантних лентивірусів, що містять послідовності shRNA для протеїну Ruk/CIN85. Лентивірусні вектори для пригнічення експресії Ruk/CIN85 методом РНК-інтерференції (pLKO.1-shRuk/CIN85 R19-23) було отримано шляхом лігування відповідних олігонуклеотидів після їх відпалювання (для R22 5′-CCGGCCAGCAGAAACGAGAGATTAACTCGAGTTAATCTCTCGTTTCTGCTGG-3′ та 5′-AATTCAAAAACCAGCAGAAACGAGAGATTAACTCGAGTTAATCTCTCGTTTCTGCTGG-3′) у вектор pLKO.1 puro (Addgene, США) за центрами пізнавання для ендонуклеаз рестрикції EcoRI і AgeI. Отримані плазміди були нароблені й очищені з клітин *E. coli* з використанням набору для очищення плазмідної ДНК (NucleoSpin plasmid purification kit, Machery-Nagel, Німеччина). Лентивіруси, що містять Ruk/CIN85 shRNA або scrambled shRNA, одержали шляхом котрансфекції «пакувальних» клітин HEK293T векторами pLKO.1-R19-23 або pLKO.1-scrambled, вектором pMD2.G, що кодує вірусний протеїн капсиду VSV-G (Vesicular stomatitis virus G glycoprotein), та пакувальним вектором psPAX2, що кодує вірусні протеїни gag і pol. Вірус збирали на 36-ту, 48-му і 60-ту год після трансфекції. Після інфікування відповідними лентивірусами клітини пасажували кілька разів за присутності селективного антибіотика пуроміцину без подальшого субклонування.

Вестерн-блот-аналіз. Для дослідження вмісту протеїнів клітини висівали на чашки Петрі (діаметром 6 см). Через 24 год до клітин додавали суспензію, яка містила лентивірусні частинки, за присутності катіонного полімеру полібрену (10 мкг/мл), що забезпечувало підвищення ефективності інфікування клітин. Через 24 год середовище змінювали на свіже, що містило селективний антибіотик пуроміцин (1 мкг/мл). Клітини лізували та проводили вестерн-блот-аналіз, як описано у [5] з використанням поліклональних антитіл кроля до Ruk/CIN85 (1:3000) [9] та до β-актину (1:5000) (Sigma). Імунореактивні смуги детектували за допомогою системи для підсиленої хемілюмінесценції (ECL Western Blotting System, Amersham, Велика Британія). Інтенсивність отриманих сигналів аналізували за допомогою денситометрії з використанням програми ImageJ. Рівень експресії β-актину визначали для підтвердження однакової кількості протеїну в пробах.

Дослідження рухливості клітин методом «заростання подряпини» у клітинному моношарі. Для проведення експериментів клітини лінії MCF-7 wt та зі стабільною надекспресією Ruk/CIN85 (сублінія G4) висівали у 6-лункові планшети і вирощували до 80% конфлюенту в середовищі DMEM, що містило 10% FBS. Через 24 год після висівання клітини інфікували лентивірусами, які експресували Ruk/CIN85-shRNA або scrambled shRNA. Подряпину у клітинному моношарі робили за допомогою носика на 10 мкл до автоматичних піпеток. Після цього середовище культивування замінювали на свіже, що містило 1 мкМ мітоміцину С (Sigma) для усунення клітинної проліферації. Через 24 год ефективність заростання подряпини аналізували за допомогою

фазово-контрастного мікроскопа. Відстань, на яку мігрували клітини, вимірювали за допомогою програмного забезпечення QuickPHOTO Camera 2.2.

Протокова цитофлуориметрія. Для оцінювання вмісту ABCG2-транспортерів клітини лінії MCF-7 wt зі стабільною надекспресією Ruk/CIN85 (сублінія G4) та клітини сублінії G4, інфіковані рекомбінантним лентивірусом R22 і відібрані за присутності пуроміцину ($2 \cdot 10^6$ клітин на пробу), фіксували в 96%-му розчині етанолу протягом 30 хв. Після оброблення суспензії 0,1%-м розчином тритону X-100 клітинну суспензію інкубували з антитілами до ABCG2-транспортера (Abcam) протягом 45 хв за кімнатної температури з подальшим відмиванням PBS. Антимишачі антитіла, мічені AlexaFluor488 (Invitrogen), використовували як вторинні. Клітини аналізували на протоковому цитофлуориметрі COULTER EPICS XL™ (Beckman Coulter).

Оцінювання метаболічної активності клітин з використанням MTT. Активність електронотранспортного ланцюга як показника кількості живих клітин оцінювали за швидкістю відновлення MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) у клітинах LLC, як описано в роботі [10]. Реакцію проводили у 96-лункових планшетах. Кількість клітин LLC у лунці становила $1 \cdot 10^4$ в об'ємі 200 мкл. У кожну лунку додавали 20 мкл розчину MTT (4 мг/мл у PBS) та інкубували протягом 2 год при 37 °C. Після інкубації планшети центрифугували (600 *g*, 7 хв) і зберігали їх у темряві за 4 °C упродовж 20 год. Осад формазану розчиняли у 150 мкл диметилсульфоксиду. Через 15 хв вимірювали екстинкцію на цифровому спектрофотометрі µQuant (BioTEK, США) за $\lambda = 570$ нм. Метаболічну активність клітин подавали у відсотках відносно контролю.

Визначення продукції АФК. Внутрішньоклітинні рівні АФК аналізували методом хемілюмінесценції з використанням люмінолу, як описано в [11]. Клітини HT-29 вирощували до 80% конфлюенту у середовищі DMEM, що містило 10% FBS. Далі клітини обробляли розчином акутази (1 хв при 37 °C), промивали PBS (без $MgCl_2$ і $CaCl_2$), осаджували центрифугуванням (1000 *g* протягом 5 хв) та ресуспендували в холодному розчині HBSS (з $MgCl_2$ і $CaCl_2$). Потім $2 \cdot 10^5$ клітин HT-29 висівали в кожну лунку 96-лункового планшета. До кожної лунки додавали 250 мкМ люмінолу та 1 U пероксидази хрону (кінцеві концентрації) в загальному об'ємі 200 мкл. Хемілюмінесценцію вимірювали за допомогою люмінометра FLx800 (BioTek, США) через 5 хв після додавання суміші «пероксидаза хрону/люмінол» протягом 30 хв з інтервалом 1 хв за кімнатної температури.

Статистична обробка результатів. Статистичну обробку результатів проводили у програмі Excel 2007. Експериментальні результати подано як середнє арифметичне та стандартне відхилення вибірки. Достовірність змін оцінювали за t-критерієм Стьюдента.

Результати та обговорення

Експресія shRNA, що пригнічує експресію адаптерного протеїну Ruk/CIN85, у клітинах ссавців

Послідовності shRNA, що гіпотетично можуть пригнічувати експресію Ruk/CIN85, було запропоновано компанією Open Biosystems (Thermo Fisher Scientific, Inc, США). Усього було перевірено п'ять послідовностей: R19 (2435–2455), R20 (1775–1795), R21 (561–581), R22 (2175–2195) та R23 (1082–1102) (у дужках позначено позиції відповідних нуклеотидів на мРНК Ruk/CIN85, загальна довжина якої становить 3348 п. н.). Із цих послідовностей R20, R21, R22 та R23 локалізовані у протеїнокодувальній ділянці мРНК (рис. 1), а R19 — у 3′-нетрансльованій ділянці мРНК. Лентивірусні вектори для пригнічення експресії Ruk/CIN85 методом РНК-інтерференції (pLKO.1-shRuk/CIN85 R19-23) одержували, як описано в розділі «Матеріали і методи». Схему будови однієї з пар олігонуклеотидів (R22) наведено на рис. 1, *Б*.

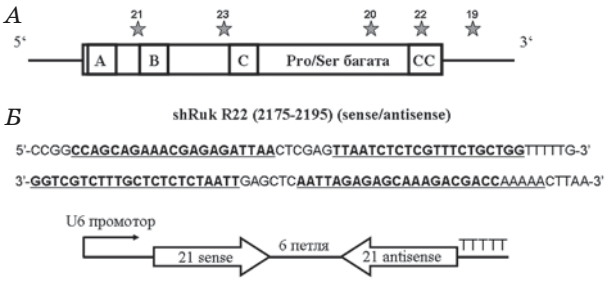

Рис. 1. **Структурна організація повнорозмірного варіанта адаптерного протеїну Ruk/CIN85:** показано SH3A-, SH3B- та SH3C-домени, Pro/Ser-багату ділянку та суперспіралізований (CC) район. Зірочками позначено ділянки протеїну, яким відповідають гіпотетичні shRNA-послідовності для РНК-інтерференції (R20, R21, R22, R23). Послідовність R19 комплементарна до 3′-нетрансльованої ділянки мРНК Ruk/CIN85

Геном лентивірусів містить три гени, що розташовані в геномній РНК у порядку 5′-gag-pol-env-3′, а також допоміжні гени. З міркувань безпеки лентивірусні вектори, у тому числі pLKO.1 puro, ніколи не несуть гени, необхідні для їх реплікації. Для одержання віріонів клітини «пакувальної» клітинної лінії HEK293T, які експресують великий Т-антиген вірусу SV40, трансфікували трьома векторами — лентивірусним (pLKO.1), що містить послідовність для інтеграції в геном, пакувальним (psPAX2), який кодує протеїни gag і pol, та вектором, що кодує протеїн капсиду (pMD2.G) (рис. 2). Супернатант, що містить вірусні частинки, збирали перший раз через 36 год після трансфекції, а потім ще 2 рази кожні 12 год.

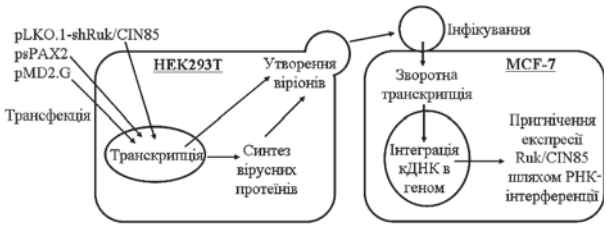

Рис. 2. **Схема зараження «клітин-мішеней» лінії MCF-7 лентивірусами, одержаними в «пакувальних» клітинах HEK293T**

Було отримано п'ять різних лентивірусів на основі pLKO.1puro, що містили потенційні послідовності анти-Ruk/CIN85 shRNA (R19-23). Ці віруси додавали (за присутності полібрену) до клітин-«мішеней», зокрема до гліоми С6, яка характеризується високим ендогенним вмістом Ruk/CIN85, та аденокарциноми грудної залози людини MCF-7 зі стабільною надекспресією Ruk/CIN85 (сублінія G4). Експресію Ruk/CIN85 у лізатах відповідних клітин досліджували за допомогою вестерн-блот-аналізу. Виявили, що одна з потенційних послідовностей, а саме R22, пригнічує експресію гена Ruk/CIN85 як у клітинах С6 (рис. 3, *А*), так і в клітинах лінії MCF-7 (рис. 3, *Б*).

Дослідження впливу пригнічення експресії адаптерного протеїну Ruk/CIN85 на клітинну міграцію, проліферацію, утворення АФК та експресію транспортера ABCG2 в пухлинних клітинах

Подальші дослідження впливу пригнічення експресії Ruk/CIN85 на біологічні властивості клітин здійснювали за допомогою експериментального підходу, що його широко використовують для порівняльного аналізу міграційного потенціалу клітин — заростання подряпини у клітинному моношарі. Інфікування клітин G4 лентивірусами, що містили Ruk/CIN85 shRNA (R22), але не scrambled shRNA, супроводжувалося зниженням експресії Ruk/CIN85 приблизно на 70% (рис. 4, *А*). Через 24 год ефективність заростання подряпини аналізували за допомогою фазово-контрастного мікроскопа.

За результатами, наведеними на рис. 4, рухливість контрольних клітин була приблизно на 40% нижчою порівняно з клітинами субклону G4. Виявлено, що пригнічення експресії Ruk/CIN85 призводило до подальшого посилення рухливості клітин G4 ще на 40% порівняно з контрольними клітинами (рис. 4, *Б, В*). Наші дані, отримані на клітинах слаботрансформованої лінії аденокарциноми грудної залози людини MCF-7 за умов надекспресії адаптерного протеїну Ruk/CIN85, відрізняються від результатів, одержаних з використанням високоінвазивних ліній раку грудної залози людини MDA-MB-231, MDA-MB-435s та Hs578T. У цих лініях siRNA-опосередковане пригнічення експресії ендогенного Ruk/CIN85 зумовлювало інгібування інвазивних властивостей клітин в Matrigel assay [12].

Відомо, що клітини, які зазнають злоякісної трансформації, характеризуються підвищеною резистентністю до лікарських препаратів. Важливим чинником, що визначає множинну резистентність до лікарських препаратів під час хіміотерапії онкологічних захворювань, є транспортер ABCG2 [8].

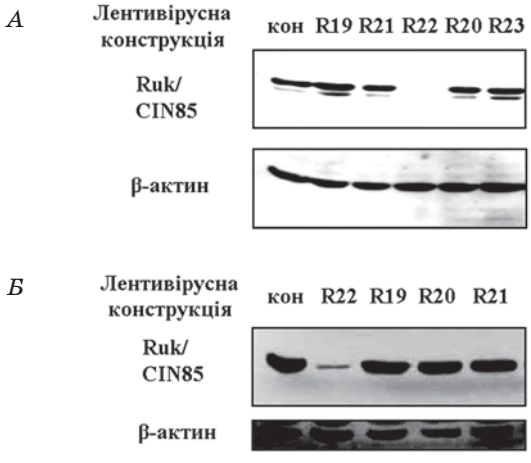

Рис. 3. **Аналіз експресії Ruk/CIN85 у клітинах гліоми щура С6 (*А*) та в клітинах аденокарциноми грудної залози людини лінії MCF-7 з надекспресією Ruk/CIN85 (сублінія G4) (*Б*) після оброблення лентивірусами pLKO.1puro R19-23:**
клітини, інфіковані scrambled shRNA, використовували як контроль. Вміст протеїну визначали за допомогою вестерн-блот-аналізу

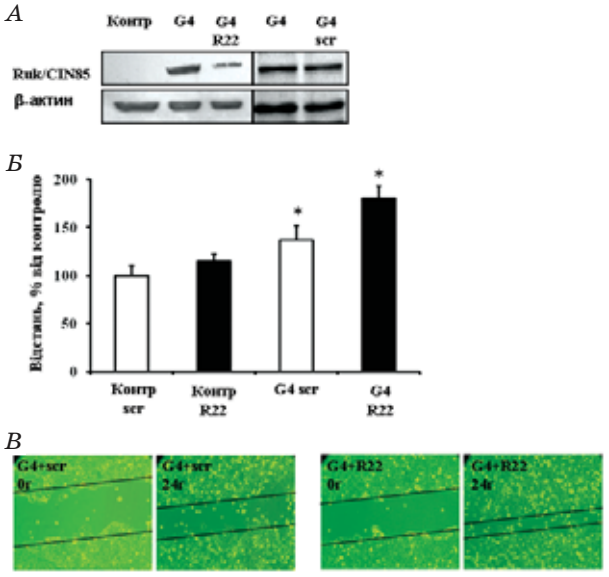

Рис. 4. **Вестерн-блот-аналіз вмісту Ruk/CIN85 у клітинах лінії MCF-7 «дикого типу» (контроль), клітинах сублінії G4 і клітинах G4, інфікованих лентивірусами, що експресують Ruk/CIN85 shRNA R22 або scrambled shRNA (*A*). Субконфлюентні моношари клітин лінії MCF-7 «дикого типу» (контроль) або клітин сублінії G4 з високим рівнем стабільної надекспресії Ruk/CIN85 інфікували лентивірусами, що експресували Ruk/CIN85 shRNA R22 або scrambled shRNA (*Б*).**

Подано середні дані з чотирьох експериментів ± SEM. * Статистично значуща відмінність показників клітин сублінії G4 від контрольних за парного порівняння за t-критерієм Стьюдента і рівнем значущості $P \leq 0{,}05$.

Типові фотографії зон подряпини перед і після 24 год заростання: показано клітини сублінії G4, інфіковані лентивірусами, що експресують Ruk/CIN85 shRNA R22 або scrambled shRNA (*B*)

Для того, щоб дослідити зміни в рівні експресії цього транспортера в клітинах MCF-7 з надекспресією Ruk/CIN85, застосовували метод протокової цитофлуориметрії. Було виявлено, що близько 37% клітин сублінії G4 експресують ABCG2 порівняно з 13% контрольних клітин MCF-7 (рис. 5). Пригнічення експресії Ruk/CIN85 призводило до зниження експресії ABCG2 до рівнів, що суттєво не відрізнялись від значень у контрольних клітинах. Одержані дані свідчать про потенційну роль досліджуваного адаптерного протеїну в розвиткові резистентності до протипухлинних препаратів.

Аби виявити, які ефекти спричинює пригнічення ендогенних рівнів експресії Ruk/CIN85, а також для оцінювання здатності одержаних лентивірусів інфікувати клітини різних типів, було використано клітини мишачої карциноми легені Льюїса (LLC) та аденокарциноми ободової кишки людини HT-29. За допомогою МТ-тесту встановили, що через 24 год після висівання кількість клітин із пригніченою експресією Ruk/CIN85 була майже вдвічі вищою порівняно з контрольними клітинами, що свідчить про посилення проліферації клітин LLC за умов пригнічення експресії ендогенного протеїну Ruk/CIN85 (рис. 6). Ці результати загалом узгоджуються з даними щодо клітин лінії MCF-7, для яких показано повільнішу проліферацію за умов надекспресії Ruk/CIN85 [5].

Було виявлено, що повне пригнічення експресії Ruk/CIN85 призводило до зниження рівня утворення АФК в клітинах HT-29 приблизно на 72% від значень у контрольних клітинах (рис. 7). Наскільки нам відомо, роль Ruk/CIN85 у регулюванні продукції АФК раніше не було описано.

Оскільки зростання активності дихального ланцюга мітохондрій супроводжується підвищеним утворенням АФК, які є побічним продуктом цієї реакції, дані про те, що пригнічення експресії Ruk/CIN85 водночас призводить до посилення проліферації та зниження рівнів АФК, можуть видатися суперечливими. Проте слід мати на увазі, що важливим джерелом АФК у клітинах є протеїни плазматичної мембрани, зокрема НАДФН-оксидази [13]. Наші попередні дані вказують на те, що Ruk/CIN85 може брати участь у регулюванні НАДФН-оксидазної активності, проте підтвердження цієї гіпотези потребує подальших досліджень.

Таким чином, було одержано лентивірусну конструкцію R22, що пригнічує експресію Ruk/CIN85 у клітинах C6, HT-29, LLC, а також в сублінії клітин MCF-7 з надекспресією цього протеїну (G4). Результати експресії транспортера ABCG2 в клітинах аденокарциноми грудної залози людини лінії MCF-7 свідчать про послаблення однієї з ознак, характерних для злоякісної трансформації, за зниження рівня Ruk/CIN85. Дещо несподіваними виявилися результати стосовно вищої рухливості клітин G4 зі зниженим вмістом Ruk/CIN85 порівняно з контрольними клітинами. Це можна пояснити тим, що рівень експресії Ruk/CIN85 у клітинах G4, інфікованих специфічним лентивірусом, ще залишався достатньо високим порівняно з контрольними клітинами лінії MCF-7. На сьогодні добре відомо, що адаптерні/риштувальні протеїни регулюють інтенсивність вихідного сигналу у концентраційно-залежний спосіб. Це означає, що

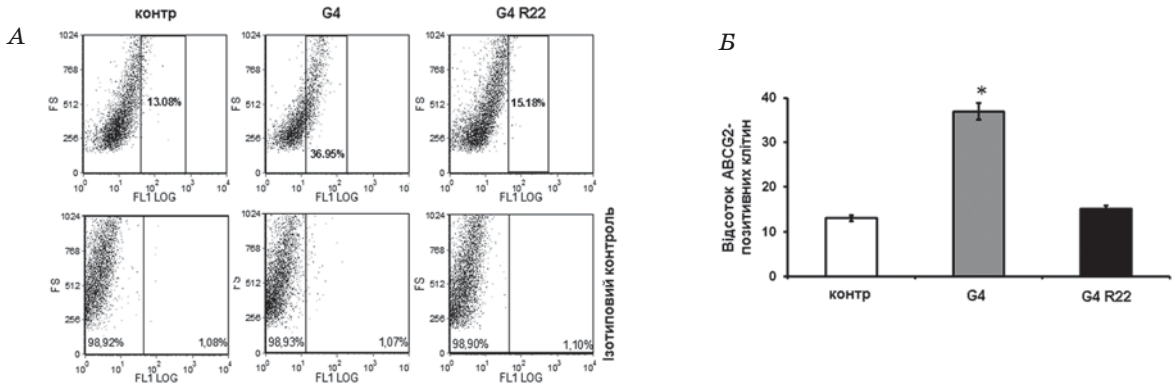

Рис. 5. **FACS (fluorescence-activated cell sorting)- аналіз експресії мембранного транспортера ABCG2 у контрольних клітинах лінії MCF-7, клітинах сублінії G4 та клітинах G4 з пригніченою експресією Ruk/CIN85:**
А — точкова (dotplot) гістограма експресії ABCG2; *Б* — частка (у відсотках) ABCG2-позитивних клітин від загальної кількості досліджуваних клітин.
* Тут і далі — статистично значуща відмінність показників клітин сублінії G4 від контрольних за парного порівняння за t-критерієм Стьюдента і рівнем значущості $P \leq 0{,}05$

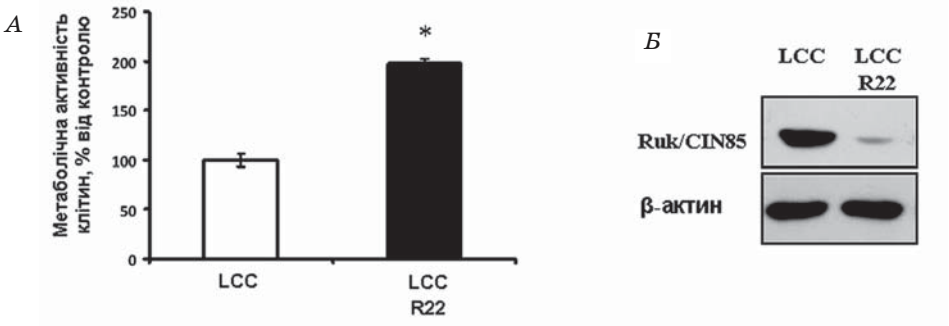

Рис. 6. **Аналіз проліферативної активності контрольних клітин LLC та клітин LLC зі зниженою експресією Ruk/CIN85 за метаболічною активністю, визначеною з використанням MTT:**
А — метаболічну активність клітин з пригніченою експресією Ruk/CIN85 подавали у відсотках відносно активності контрольних клітин, яку було прийнято за 100% . Наведено середні дані з трьох експериментів ± SEM;
Б — вестерн-блот-аналіз експресії Ruk/CIN85 у контрольних клітинах LLC та клітинах, інфікованих лентивірусами, що експресують Ruk/CIN85 shRNA R22

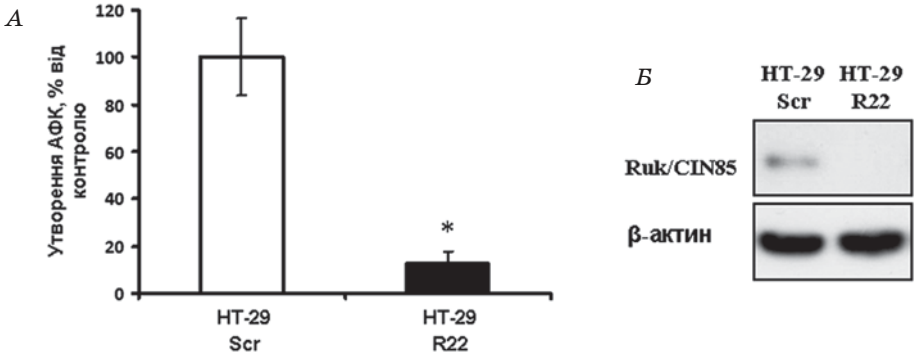

Рис. 7. **Аналіз утворення АФК у контрольних клітинах HT-29 та клітинах зі зниженою експресією Ruk/CIN85 методом хемілюмінесценції з використанням люмінолу та пероксидази:**
А — утворення АФК в клітинах з пригніченою експресією Ruk/CIN85 виражали у відсотках відносно активності контрольних клітин, яку було прийнято за 100% . Наведено середні дані з трьох експериментів ± SEM. *Б* — вестерн-блот-аналіз експресії Ruk/CIN85 у контрольних клітинах HT-29 та в клітинах, інфікованих лентивірусами, що експресують Ruk/CIN85 shRNA R22

надекспресія Ruk/CIN85 вище стехіометричного оптимуму може призводити до домінантно-негативного ефекту [14, 15]. Можна припустити, що «оптимальна» концентрація Ruk/CIN85, за якої рухливість клітин є максимальною, перевищує рівень контрольних клітин MCF-7, але нижча за рівень у клітинах G4.

Отже, результати вивчення проліферативної активності клітин мишачої карциноми легень LLC та утворення АФК клітинами аденокарциноми ободової кишки людини HT-29 свідчать про те, що зниження ендогенних рівнів Ruk/CIN85 змінює біологічні властивості зазначених типів клітин. Для того, щоб краще зрозуміти роль адаптерного протеїну Ruk/CIN85 у регулюванні біологічних відповідей пухлинних клітин, необхідні подальші дослідження.

Роботу здійснено за фінансової підтримки цільової комплексної міждисциплінарної програми наукових досліджень НАН України «Фундаментальні основи молекулярних та клітинних біотехнологій», а також спільного українсько-російського гранту між НАН України та РФФД 2012 р.

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ОТВЕТ ОПУХОЛЕВЫХ КЛЕТОК НА ПОДАВЛЕНИЕ ЭКСПРЕССИИ АДАПТЕРНОГО ПРОТЕИНА RUK/CIN85 РЕКОМБИНАНТНЫМИ ЛЕНТИВИРУСАМИ

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Ruk/CIN85 является одним из адаптерных протеинов, играющих важную роль в регулировании процессов пролиферации, подвижности и гибели клетки. Недавно нами было показано, что сверхэкспрессия Ruk/CIN85 усиливает онкогенный потенциал клеток аденокарциномы грудной железы человека линии MCF-7. Цель данного исследования — установить, влияет ли на свойства клеток подавление экспрессии Ruk/CIN85. Для Ruk/CIN85 РНК-интерференции были получены лентивирусные конструкции, содержащие Ruk/CIN85-специфические последовательности small hairpin RNA. С помощью полученных рекомбинантных лентивирусов установлено, что подавление экспрессии Ruk/CIN85 влияет на биологические свойства (подвижность, пролиферацию, уровень экспрессии транспортера ABCG2, образование активных форм кислорода) опухолевых клеток разных типов, а именно: аденокарциномы грудной железы человека линии MCF-7, аденокарциномы ободочной кишки человека HT-29 и мышиной карциномы легких Льюиса.

Ключевые слова: адаптерные протеины, Ruk/CIN85, РНК-интерференция, лентивирусные векторы, подвижность клеток, активные формы кислорода.

RECOMBINANT LENTIVIRUS-MEDIATED SILENCING OF ADAPTOR PROTEIN RUK/CIN85 EXPRESSION INFLUENCES BIOLOGICAL RESPONSES OF TUMOR CELLS

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Ruk/CIN85 is an adaptor protein that plays important roles in the regulation of cellular processes such as cell death, proliferation and motility. It was recently shown that overexpression of Ruk/CIN85 increases the oncogenic potential of human breast adenocarcinoma MCF-7 cells. It was the aim of the present study to investigate whether inhibition of Ruk/CIN85 expression has an effect on the biological properties of the cells. In order to down-regulate Ruk/CIN85 expression of small interfering RNA-based approach was used. For down-regulation of Ruk/CIN85 lentiviral constructs encoding Ruk/CIN85-specific small hairpin RNA sequences were generated. By using the obtained recombinant lentiviruses it was shown that inhibition of Ruk/CIN85 expression influences biological properties (motility, proliferation, ABCG2 expression, and ROS generation) of various tumour cell types such as human breast adenocarcinoma MCF-7, human colorectal adenocarcinoma HT-29, and Lewis mouse lung carcinoma cells.

Key words: adapter proteins, Ruk/CIN85, RNA-interference, lentiviral vectors, cell motility, active oxygen forms.

ПЕРЕДПЛАТА — 2013

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