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SELECTED TOPICS IN MICROBIOLOGY

CONTENTS

FOREWORD

Selected topics in microbiology, S. Koren, V. Kotnik, M. Likar II-1

ARTICLES

Trends in virology, M. Likar II-3

Hantavirus genomic variation and disease distribution, T. Avšič-Županc, M. Poljak II-7

Typhoid - microbiology and public health, B. Cvjetanović II-11

Interferons in antitumor therapy: Current status and future prospects, W. R. Fleischmann Jr. II-15

The role of interferons in the regulation of cell function and in the pathogenesis of disease, R. Mažuran II-19

Tumor studies using tumor cells resistant to interferon, C. M. Fleischmann II-23

Chronobiology of the interferon system, S. Koren, W. R. Fleischmann Jr. II-27

Porcine interferon Gamma (PoIFN Gamma), B. Filipič, S. Rozman, K. Carlsson, A. Cencič II-31

Tumor necrosis factor - biological characteristics and applications in cancer therapy, G. Serša II-35

Production of recombinant human tumor necrosis factor α in two different Escherichia coli expression systems, V. Menart, V. Gaberc-Porekar, N. Kraševec, S. Miličić, R. Komel II-43

Natural and artificial immunomodulation, V. Kotnik, M. Čižek-Sajko, A. Ihan, S. Simčič, B. Wraber, A. Štalc II-49

Cell surface molecules that regulate ability of NK cells to kill their targets, A. Ihan II-53

Modulation of cytokine synthesis in human mononuclear cell cultures of different origin, B. Wraber II-57

Modulation of neutrophil oxidative burst, S. Simčič, F. Bobanović II-63

In vivo interference of azithromycin with some human immune functions, J. Tomažič, V. Kotnik, S. Simčič, B. Wraber II-69

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Foreword/Predgovor

The Institute of Microbiology
at the Medical Faculty in Ljubljana
organised a scientific meeting entitled

SELECTED TOPICS IN MICROBIOLOGY

which was held in Ljubljana on June 10 and 11, 1993. The symposium was a celebration of the forty-two years of distinguished work in microbiology by Prof. Miha Likar on the occasion of his retirement. Over thirty leading scientists in different fields of microbiology and immunology reviewed their past and recent experimental work. Important problems concerning emerging and re-emerging bacterial and viral diseases were discussed.

The meeting was held at a time when new methods in molecular biology had advanced the research and diagnosis of microbial infections and diseases beyond previous expectations. The polymerase chain reaction (PCR) is obviously a highly important method with a broad possibility of utilization not only in medicine but also in other natural sciences. It has been introduced to study the variation and relatedness among different virus isolates at the molecular level, and holds great potential for future molecular epidemiological studies. The structure and genetics of human immunodeficiency virus (HIV), Muerto Canyon virus (a newly discovered hantavirus) and some other newly emerging microbes have been elucidated in a shortest possible time due to new methods. New facts are constantly brought to light.

The current status and future prospects in chronobiology, immunomodulation, and cytokine therapy were also presented. Interferons and tumor necrosis factor (TNF) are among the most interesting cytokines with pleiotropic functions. Recent advances in basic research and clinical uses of interferons and TNF were specially emphasized. Laboratory production of interferons and TNF with recombinant DNA technology was described. Also, a number of possible methods for increasing the therapeutic index of interferons and TNF were discussed in detail.

Some topics presented at the meeting were related to the theoretical and practical approaches to the problem of therapeutical use of natural and man-made products for the modulation of the immune response. More and more evidence has been collected in recent years to give hope that severe diseases can be cured or at least relieved with the use of immunomodulation.

The international meeting with participants from USA, France, Austria, Croatia and Slovenia was a demonstration of successful collaboration among scientists from different laboratories with a common goal: to gain further insight into microbiological and epidemiological problems which are topical around the globe. Fruitful discussions among the international scientists led to an important exchange of information and ideas that will help to achieve this goal.

Organizing Committee:
Srečko Koren, Vladimir Kotnik, Miha Likar

The meeting was sponsored by Krka and Labormed.

Inštitut za mikrobiologijo
Medicinske fakultete v Ljubljani
je organiziral znanstveno srečanje

IZBRANA POGLAVJA IZ MIKROBIOLOGIJE

ki je potekalo v Ljubljani 10. in 11. junija 1993. Simpozij je bil posvečen prof.dr. Mihi Likarju, vodilnemu slovenskemu mikrobiologu (virologu), ki se je upokojil po dvainštiridesetih letih uspešnega in plodnega dela.

Več kot trideset pomembnih raziskovalcev na različnih področjih mikrobiologije in imunologije je predstavilo pregled svojih raziskovalnih dosežkov iz obdobja nekaj zadnjih let. Nadrobno so bile obravnavane nekatere nove, do nedavnega neznane mikrobne okužbe, kakor tudi tiste, ki so znane že dalj časa, pa ponovno pridobivajo na pomenu.

Srečanje je potekalo v obdobju, ko so sodobne molekularnobiološke metode omogočile nesluten napredek v odkrivanju in prepoznavanju mikrobnih okužb in bolezni. Verižna reakcija s polimerazo, ki se je uveljavila v medicini in v drugih naravoslovnih področjih, je bistveno izboljšala možnosti preučevanja genetskih lastnosti mikroorganizmov in odpira nova poglavja molekularne epidemiologije. Z uporabo teh in še nekaterih drugih metod so izjemno hitro in natančno določili gensko zgradbo humanega virusa imunske pomanjkljivosti (HIV) in pred kratkim odkritega virusa Muerto Canyon iz skupine hantavirusov.

Predstavljeno je bilo tudi trenutno stanje in izgledi za prihodnost na področju kronobiologije, imunomodulacije in terapije s citokini. Interferoni in dejavnik, ki povzroča nekrozo tumorjev (TNF), sodijo med najzanimivejše citokine z raznovrstnimi učinki. Opisane so bile raziskave, ki skušajo pojasniti mehanizme njihovega delovanja in povečati njihov terapevtski učinek oz. izboljšati klinično učinkovitost in uporabnost.

V okviru srečanja so bile obravnavane tudi posamezne imunološke teme, s poudarkom na teoretičnih in praktičnih problemih modulacije imunskega odziva. V zadnjih letih se je nabralo precej podatkov, ki nakazujejo, da bo imunomodulacija odigrala pomembno vlogo pri zdravljenju različnih bolezni.

Na srečanju so sodelovali znanstveniki iz ZDA, Francije, Avstrije, Hrvaške in Slovenije, ki so v zanimivih in koristnih razpravah izmenjali svoje izkušnje in poglede na različne mikrobiološke in epidemiološke probleme, ki zadevajo številne države po vsej zemeljski obli. Mnogi med njimi združujejo raziskovalne moči že dlje časa na skupnih, mednarodnih projektih in za te je bilo srečanje le ena izmed neposrednih oblik sodelovanja. Pričakovati je, da se bo takšno in podobno trajno sodelovanje med različnimi laboratoriji uspešno nadaljevalo tudi v prihodnje.

Organizacijski odbor:
Srečko Koren, Vladimir Kotnik, Miha Likar

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Pregledni prispevek/Review article

GIBANJA V VIROLOGIJI

TRENDS IN VIROLOGY

Miha Likar

Inštitut za mikrobiologijo, Medicinska fakulteta,Zaloška 7, 61105 Ljubljana, Slovenija

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Ključne besede: *virus hepatitisa C; virus Norwalk; sekvence nukleotidov popolnih virusnih genomov; PCR; genski transfer; anti HIV vakcina; virusi in rak; antivirusna zdravila*

Izvleček – Izhodišče. *Avtor poskuša prikazati na nekaterih primerih, ki jih je zbral iz mnogoštevilnih novosti v virologiji, da je molekularna biologija združila posamezne veje virologije. Nadrobno opisuje odkrivanje virusa hepatitisa C, opozarja na virus Norwalk in agens bolezni Borna. Poskuša prikazati pomen določanja popolnih sekvenc nukleotidov genomov virusov vakcinij, citomegalovirusa in rdečk.*

Zaključki. *Očitno je avtorju posebno pomemben test PCR in išče motive Nobelovca Mullisa, ki je test izumil. Pri opisu genskega transferja opisuje poskuse zdraviti z metodo cistično fibrozo in navaja, kjer poskuse opravljajo z dovoljenjem FDA. Iskanje vakcine proti aidsu predstavlja kot osrednje vprašanje današnje virologije in razmišlja o vzrokih za počasen napredek. O vlogi virusov pri raku opozarja na skupino papilomavirusov, ki so v zadnjih letih postali izredno zanimivi, kajti ni dvoma, da so navzoči pri mnogih anogenitalnih tumorjih človeka. Opisani sta »dobra« in »slaba« novica o antivirusnih zdravilih.*

Key words: *hepatitis virus C; virus Norwalk; nucleotide sequences of whole viral genoms; PCR; gen transfer; anti HIV vaccine; viruses and cancer; antiviral therapy*

Abstract – Background. *Author is trying to demonstrate that molecular virology has succeded to unite all special branches of virology. In details the discovery of hepatitis virus C is presented and the importance of the new hepatitis virus is outlined. As a new development the classification of Norwalk virus and Borna agents is mentioned and their importance for medicine stressed. Sequencing of whole viral genoms is described and some efforts to obtain them with vaccinia viruses are given.*

Conclusions. *PCR is obviously a highly important advance as the possibilities are vaste not only for medicine but also for a number of other natural sciences such as paleontology, forensic medicine and others. A description of Mullise's considerations in discovering PCR are given as described by Mullis himself. Gen transfer is the next astonishing development of medical virology and the use of some atenuated adenoviruses with integrated gen for cystic fibrosis is described in general terms. The search for an effective anti HIV vaccine is represented as a central issue in todays virology and some disappointements are outlined. Viruses and cancer is the last theme author is trying to explain admitting that the problem is not simple but complex. A case of »good« and a case of »bad« news for antiviral therapy are described.*

Uvod

André Lwoff, ki je pred nekaj več kot desetimi leti dobil Nobelovo nagrado za odkritje stopnje profaga v bakterijah, je pred nekaj leti na mednarodnem virološkem kongresu z rahlo nostalgijo opisoval »vdor biokemikov in fizikov v virologijo« (1). Že tedaj se je nadejal novosti, češ nemara pa bo molekularna biologija virologijo spet združila v celostno vedo. Tudi nam se je pred leti zdelo, da bo medicinska virologija postala samostojna veja virologije. Medicinska virologija je v tistih letih veljala v očeh molekularnih virologov kot nekaj, kar je le napol znanost, kajti medicinske viroloģe so zanimali predvsem praktični uspehi molekularne biologije, ki pa jih do nedavnega ni bilo veliko, čeprav so se že začele pojavljati rekombinantne vakcine in drugi pridelki, uporabljivi za diagnozo virusnih okužb.

Novi virusi

V zadnjih letih so osamili in identificirali več novih virusov, za katere so že sumili, da povzročajo pri človeku bolezni. Dober primer je virus hepatitisa C. Po odkritju virusa hepatitisa B v 1960-ih letih in odkritju virusa hepatitisa A v 1970-ih letih še vedno ni bilo mogoče uvrstiti precejšnjega odstotka infekcijskega hepatitisa. Vedeli smo le, da približno 90% posttransfuzijskih hepatitisov povzroča neki virus, ki smo ga imenovali neA-neB hepatitis.

Raziskovalci tvrdke Chiron v Združenih državah so že od začetka 1980-ih let vlagali velika sredstva, da bi identificirali neA-neB virus hepatitisa. Iz plazme šimpanza, ki je vsebovala velike količine infekcijskega neA-neB agensa, so osamili vso nukleinsko kislino z ultracentrifugiranjem. Po denaturiranju RNA so dobili komplementarno DNA in jo s pomočjo bakteriofaga (lambda GT-11) klonirali v Escherichia coli (2). Približno milijon komplementarnih DNA klonov so testirali s serumi pacientov s kroničnim neA-neB hepatitisom. Leta 1988 so dobili klon (klon 5-1-1), ki je kodiral specifičen protein in s hibridizacijo zgradili enovijačno RNA s 5000 do 10.000 nukleotidov. Tako so identificirali popolno sekvenco RNA genoma virusa hepatitisa C.

Že s pomočjo tega genoma so sestavili zanesljive serološke teste, encimsko imunski test in verižno reakcijo s polimerazo. Izsledki so bili osupljivi: infekcija z virusom hepatitisa C povzroči pri 80% kroničnega vironosca. Pri vseh vironoscih pa so našli ne glede na jetrne funkcijske teste histološka znamenja kroničnega hepatitisa in/ali ciroze. Podobno kot infekcija z virusom hepatitisa B je tudi infekcija z virusom hepatitisa C povezana z nastankom jetrnega karcinoma. Večina vironoscev se inficira parenteralno: z intravensko uporabo drog, krvno transfuzijo ali tetoažo. V veliki nevarnosti so zlasti uživalci drog in hemofiliki. Spolno prenašanje pa pri virusu hepatitisa C nima večje vloge. Zdravljenje z antivirusnimi zdravili sicer izboljša jetrne teste, vendar še ni znano, ali odpravi vironoštvo.

Strukturne primerjave virusa hepatitisa C z drugimi virusi kažejo, da ga prištevamo med flaviviruse (v to skupino prištevamo tudi virus »klopnega meningitisa«).
Leta 1990 je uspelo še identificirati nekaj: komplementarni klon DNA virusa, ki povzroča neA-neB hepatitis, ki se prenaša prek prebavil. Virus je hud javnozdravstveni problem v deželah v razvoju, kjer povzroča epidemije virusnega hepatitisa. Virus je prav posebno nevaren za nosečnice, pri katerih povzroča 20% mortaliteto. Z molekularno virološkimi metodami so podobno kot pri virusu hepatitisa C dokazali, da ga prištevamo v skupino kalicivirusov in ga poimenovali virus hepatitisa E (3).
Do tedaj še nismo poznali za človeka patogenih kalicivirusov; kmalu po uspehih z virusom hepatitisa E pa so vnovič z molekularno virološkimi metodami dokazali, da je kalicivirus tudi virus Norwalk, ki povzroča pogostne epidemije akutnega gastroenteritisa. Doslej so te epidemije v nadrobnostih zvečine opisali le v Združenih državah, vendar je vse bolj očitno, da je tudi virus Norwalk razširjen povsod po svetu. Etiološko povezanost med virusom Norwalk in gastroenteritisom pri človeku so zvečine dokazali na prostovoljcih, pri katerih so iz iztrebkov osamili virusno nukleinsko kislino za kloniranje in dobili tudi očiščene virione iz iztrebkov inficiranih prostovoljcev. Tudi virus Norwalk je virus z RNA in očitno iz skupine kalicivirusov.
Nevropsihiatre bo zanimalo, da so veterinarski molekularni virologi odkrili, da povzroča virus z enovijačno RNA bolezen Borna pri konjih (4). Za bolezen je značilno abnormalno vedenje konj in ovac. Po nekaterih poročilih naj bi imel povzročitelj bolezni Borna vlogo tudi pri človekovih nevropsihiatričnih boleznih. Menijo, da ima pri nastanku bolezni Borna vlogo tudi reakcija pozne preobčutljivosti.

Določanje vseh sekvenc nukleotidov virusnih genomov

V številnih laboratorijih poskušajo določiti vse sekvence nukleotidov virusnih genomov. Raziskave so deloma pod vplivom projekta Humani genom, ki ga je do nedavnega vodil sloviti Watson. Zdi se, da takšne raziskave prinašajo številne novosti, o katerih smo v preteklosti samo razmišljali, pa so postale dosegljive. Delne sekvence nukleotidov virusnih genomov so opisovali že zadnjih deset let, nedavno pa so uspeli identificirati vse sekvence nekaterih virusov. Zlasti so nadrobno preučili genome virusov vakcinije, citomegalovirusa in virusa rdečk.
Pri raziskavah virusa vakcinije so najbolj nadrobno preučili seva Western reserve in Copenhagen. Virus vakcinije je prototip ortopoksvirusov in je prvi poksvirus, ki je popolnoma sekvencioniran (5). Sekvence so določali pretežno z metodologijo končanja verige, pri čemer so uporabljali zaporedno sintetizirane začetne oligonukleotide (primere), da so se »plazili« ob sekvencah v plazmidih kloniranih odlomkih genoma.
DNA virusa vakcinije je linearna dvojna vijačnica z različnima strukturama na koncih. Vsa virusna DNA ima kovalentno zaprto zanko enojne vijačnice. Ob teh strukturah pa leži par večjih ponavljajočih se elementov z nasprotno usmeritvijo. V sevu Copenhagen so ti dolgi 12 kbr. Enojno vijačna DNA virusa vakcinije ima 191.737 ostankov, kar pomeni 181.535 popolne dvojno vijačne DNA in še 101 nukleotid vsake lasnice na koncih. Drugi sevi virusa vakcinije se razločujejo po pogostnosti delecij odsekov. Interpretacije sekvenc nukleotidov v genomu glede na mesto genov in glede na naravo kodiranih proteinov ponujajo fascinirajoče branje. Mogoče je razbrati vlogo nekaterih proteinov. Določili so že 189 spoznavnih kodiranih proteinov. Funkcije jim pripisujejo po že objavljenih delih in po oceni podobnosti aminokislin. Skupaj so določili vlogo nekaj manj kot 50 genov. Osupljivo pa je število genov zlasti na koncih genoma, ki še nimajo dorečenih nalog in niso bistveni za replikacijo virusa.
Očitno je, da virus vakcinije kodira številne na videz odvečne gene, ki ne vplivajo na funkcije virusa, ki jih lahko opazujemo, na primer pri replikaciji v celičnih kulturah. Virus očitno uporablja različne izmikajoče se taktike, da zmanjša svojo vidnost pred gostiteljevimi varstvenimi mehanizmi, zlasti pred imunskimi odzivi. Dokazali so na primer, da virus vakcinije inhibira delovanje sistema komplementa.
Sekvence seva Copenhagen so močno podobne doslej objavljenim sekvencam seva Western reserve, vendar so že identificirali nekatere razločke. Spreminjanja sekvence nukleotidov očitno odsevajo rekombinacijske dogodke. Prav je pripomniti, da je poreklo virusa vakcinije nejasno in se današnji sevi razločujejo v mnogih sekvencah od naravnega divjega tipa virusa vakcinije. Pojav pojasnjujejo s tem, da je Jenner cepil v času, ko so v bolnišnicah ležali številni bolniki s črnimi kozami in sta se virusa črnih koz in vakcinije (aseptike še niso poznali) rekombinirala.
DNA citomegalovirusa je bistveno večja, kot je DNA večine drugih herpesvirusov, h katerim prištevamo tudi citomegalovirus. Skupni geni s herpesvirusi so v genomu citomegalovirusa osrednje v genomu, medtem ko končne sekvence genoma niso podobne sekvencam ob koncu genomov drugih herpesvirusov. Nekatere patološke značilnosti tega virusa obeta pojasniti spoznanje, da so našli v genomu citomegalovirusa tudi gene za večje polipeptide poglavitnih molekul tkivne skladnosti razreda 1, ki so homologni receptorju limfocitov T. Spoznanje je še tem pomembnejše, ker so nekateri novejši herpesvirusi (na primer herpesvirus 6) podobnejši citomegalovirusu kot drugim herpesvirusom. Te raziskave so nedavno odkrile, da obstaja tudi herpesvirus 7. Razumljivo je, da njegovo vlogo pri človekovih boleznih še raziskujejo.
Sekvencioniranje so do kraja izpeljali tudi že za virus rubele (rdečk). Virus je član družine togavirid in edini predstavnik rodu rubivirus. Virus rubele ima enojno vijačnico RNA z 9757 baznih parov in izredno veliko količino gvanina in citozina, skoraj 70%. Študij homologije je pokazal, da sta se virus rubele in virus Sindbis (v preteklosti smo ga prištevali med arboviruse) razvila s kompleksno intervirusno rekombinacijo med razvojem (6).

Verižna reakcija s polimerazo

Pred nekaj manj kot desetimi leti je nastal nov test za dokazovanje nalezljivih in drugih bolezni, verižna reakcija s polimerazo ali PCR (po angl. = polymerase chain reaction). Test je presenetil po svoji natančnosti in občutljivosti. V medicini si je PCR silno hitro pridobila ugledno mesto med diagnostičnimi testi. Raziskovalci zvečine nekoliko zviška gledajo na diagnostične teste kot nekaj, kar ni posebno »znanstveno«. In vendar je Kary B. Mullis, ki je test sestavil, letos dobil Nobelovo nagrado (7).
Zanimivo je, kako je PCR nastala. Ko se je Mullis peljal s prijateljico za konec tedna na počitnice in se je ponoči umikal z ene polovice ceste na drugo polovico čez polno črto, se je domislil, da je DNA zgrajena podobno iz dveh vijačnic, ki se včasih razpreta in spet spojita. Bistvo je, da se ponoči na cesti lahko izogne, ker vidi luči nasproti prihajajočega avtomobila. Ali bi lahko podobno pretrgal in spet povezal dvojno vijačnico? Odsek z enojno vijačnico bi razmnoževala polimeraza, pretrganje pa bi dosegel z dvema začetnima oligonukleotidoma, ki bi bila pravilno izbrana. S segrevanjem pa bi dosegel amplifikacijo, »razmnoževanje« odseka v velikanskem številu, saj bi že dvajset ciklov dalo milijon kopij, po 30 ciklih pa bi bilo kopij 1 milijarda!
Test PCR uporabljajo danes z velikim uspehom v medicini, paleontologiji, sodni medicini in še v drugih vedah. Osupljivo je, da s PCR lahko določajo krvne skupine egipčanskih mumij. DNA je nenavadna molekula, posamezni odseki so tako odporni, da pomenijo hudo težavo za praktično uporabo testa, kajti prenesejo avtoklaviranje. Z drugimi besedami to pomeni, da kadar se laboratorij kontaminira z odseki DNA, se jih skoraj ne more več znebiti. Takšni odseki bi se umevno prikradli v teste in izsledki bi bili zmotni in zavajajoči. Mikrobiologi smo vajeni, da z avtoklavom »pometemo« vse mikroorganizme in s tem preizkuse začnemo na novo in se varujemo pred kontaminacijo z nezaželenim mikroorganizmom.
Razumljivo je, da Mullisu k odkritju PCRa ni pomagala samo nočna

vožnja ob koncu tedna. Imel je večletne izkušnje z določanjem odsekov DNA v krvi. Pri tem je uporabljal sonde, da se je razmnoževanje odsekov DNA začelo prav tam, kjer je želel. Kot pri mnogih pomembnih odkritjih je Mullisu pomagala tudi genialna zamisel, da ni vse res, kar pišejo in verjamejo znanstveniki: veljalo je, da bakterijska alkalna fosfataza prenese segrevanje pri 100°C in si po kuhanju opomore. Pomen tega encima pri PCR pa je v tem, da z njo lahko odstranimo nukleotide, ki v testu nivo zaželeni, kajti nastajajo v velikem številu. Tako je Mullis imel vse kamenčke »mozaika PCR«, v katerem je namesto bakterijske alkalne fosfataze uporabil kar polimerazo.

Mullis je svoje odkritje opisal. Piše, da je vzkliknil »eureka« in pri tem prebudil prijateljico, ki je mislila, da je zgrešil pot do počitniške hišice in sta prišla v kraj Eureka v Kaliforniji, ki je 160 km severneje. Ustavil je ob cesti in v trenutku natančno videl, kako bo delovala PCR: odlomek DNA, ki bo ograjen z dvema začetnima nukleotidoma, se bo ob vsakem segrevanju »množil« (amplificiral). Tako se je tudi zgodilo.

V začetku uporabljanja PCR so virološki laboratoriji začeli ostro tekmo, kdo bo prvi PCR uporabljal in izdelal okoliščine, v katerih trdoživost DNA ne bo delala preglavic. Pri nas smo test začeli uporabljati že zgodaj, čeprav so se mi na nekem kongresu mnogi posmehovali, češ: »Kaj, v Ljubljani boste delali PCR? Kako?« PCR že redno uporabljamo pri nejasnih in spornih izsledkih za HIV in nekatere druge viruse.

Genski transfer s posredovanjem virusov

Razumljivo je, da velja največ zanimanja za metode, s katerimi bi prenašali gene v celice sesalcev in poskušali razrešiti nekatera temeljna biološka in medicinska vprašanja.

Že nekaj let osupli spremljamo raziskave s transgenskimi mišmi (8), ki ponujajo modele za preučevanje virusne patogeneze. Zmožnost izražati gene in vivo s transgensko tehnologijo ponuja nove možnosti za preučevanje interakcije virusnih genov ali posameznega gena z gostiteljevimi celicami. Po prvi strategiji se virusni protein izrazi pod nadzorovanjem lastnega promotorja, kot je na primer pri enem od genov HIVa. Pri drugi strategiji pa se virusni protein v specializirani celici izrazi z uporabljanjem za celico specifičnega promotorja, kot je na primer podganji promotor pri beta celicah Langerhansovih otočkov trebušne slinavke ali tuji promotor za izražanje ovojničnega antigena HIVa (glikoproteina 120) v astrocitih: uspeli so na miših ponarediti nevrološke pojave pri aids demenci (9).

Zanimiva je povezanost med virusi in med od inzulina odvisnim diabetesom na modelu transgenskih miši, v katerem se je izrazil virusni protein v pankreasnih otočkih s podganjim promotorjem za inzulin. Model je omogočil opazovanje učinka samega izraženega antigena ali hkrati z induciranim antivirusnim odzivom in se pokaže šele pozneje v življenju. Virusni gen, uveden v stopnji jajca in vključen v zarodno linijo, ni povzročil diabetesa, odvisnega od inzulina, infekcija z istim ali sorodnim virusom pozneje v življenju pa je izzvala antivirusni odziv, ki selektivno uničuje beta celice. Od inzulina odvisni diabetes je nastal 14. dan, kadar se je transgen izrazil v beta celicah, in 120. do 200. dan, kadar se je izrazil v beta celicah in v priželjcu. Imunsko-kemični in genetični poskusi nakazujejo, da je za od inzulina odvisni diabetes potreben samo CD^{8+} limfocit.

Ameriška ustanova Food and Drug Administration (FDA) je že dovolila v praksi uporabiti genetično manipulirane adenoviruse za zdravljenje mukoviscidoze (cistične fibroze). Temeljni poskus je sorazmerno preprosto opisati: adenovirus s slabotno virulenco nosi ob infekciji v dihala poskusne osebe še gen za alfa 1 antitripsin, ki tem bolnikom primanjkuje in zato mladi umirajo. S takšnim zdravljenjem se danes ukvarjajo že na več ustanovah po svetu, najbolj znane pa so na univerzah v ameriških državah Michigan in Iowa. Dovoljeni so programi na National heart, lung and blood institute, kjer bodo zdravili z adenovirusom tipa 5, ki pa nima E1 genov, ki so nujni za virusno replikacijo in E3 genov, ki imunskemu sistemu preprečijo spoznati virus. Inficirali bodo 10 bolnikov, starih nad 21 let, v levo nosnico in leva pljuča. Na univerzi v Iowi pa bodo uporabili adenovirus tipa 2 brez E1 genov pa z ohranjenimi E3 geni (10).

Na univerzi v Michiganu hkrati z gensko terapijo poskušajo zdraviti še pet primerov dedne bolezni hiperholesterolemije. Zanimivo je, da pri teh že poročajo o uspehih, kajti holesterol je že po prvih »infekcijah« usahnil v krvi bolnikov skoraj za tretjino.

Pri poskusih s prenašanjem genov uporabljajo v mnogih laboratorijih mišje retroviruse, kajti ti virusi se med replikacijo za določen čas vključujejo v genom celice gostiteljice, ki za ta čas izraža nove gene, vključene kot provirus. Nekatere značilnosti naravnega cikla retrovirusa so zato idealne za genski transfer. Z razmnoževanjem specifičnih rekombinantnih genomov in specializiranih celičnih sevov, ki spodbujajo učinkovito nastajanje zrelih virusnih delcev, preprečujejo pa sproščanje in replikacijo kompetentnega virusa, je danes na voljo sistem za genski transfer, ki je bistveno učinkovitejši od vseh dosedanjih metod, ki so bile iz tehničnih vzrokov navadno mogoče le v laboratorijskih okoliščinah.

O genskem transferju ponekod neradi govorijo, ker obstajajo skupine aktivistov, ki takšnim poskusom nasprotujejo in jim obetajo nesrečen konec. Najstrožji predpisi so glede genske manipulacije v Nemčiji in danes mnogi pravijo, da so nemški molekularni biologi razvili številne nove vrste virusov, zaradi omejevalnih predpisov pa so smetano pobrali v Združenih državah, kjer ravnajo po navodilih ugledne FDA.

Anti HIV vakcina

Raziskave aidsa so dosegle obseg, ki ga virologi še nismo doživeli pri nobenem virusu ali virusnih boleznih. Mnogi se zavedajo, da je prišel čas, ko naj bi virologija prispevala vakcino, ki bi preprečevala infekcijo s HIVom, kajti vse bolj je očitno, da je upanje na zanesljivo zdravilo še bolj oddaljeno kot vakcina (11). Naj še dodamo, da je danes za virologijo na splošno, ne le za medicinsko virologijo, vakcina proti HIVu najbolj pomembna raziskovalna naloga.

Več kandidatk-vakcin proti HIVu so že preizkusili: zvečine imajo številne inaktivirane virusne delce, ki pa so izgubili tista sporočila iz genoma, ki so nujna, da nastajajo novi infektivni virioni. Že od prvih začetkov iskanja vakcine proti infekciji s HIVom pa je bilo vsem očitno, da je nujen živalski model, na katerem bi preizkušali varnost in učinkovitost vakcine. Več skupin je že pokazalo, da inaktivirane vakcine lahko zavarujejo makake opice pred infekcijo z živim SIVom (= simian immunodeficiency virus). Nekaj časa je delo na modelu z makaki lepo napredovalo (12), dokler raziskovalcev ni pretreslo spoznanje, da je opice mogoče deloma zavarovati pred HIVom tudi s celicami, v katerih HIV ni navzoč! Teoretično je to lahko deloma pojasniti (čeprav ne do kraja), kajti nekateri proteini HIVa so podobni antigenom humanih levkocitov. Raziskave na makakih so pustile neprijeten občutek: ali bomo lahko načrtovali izdelavo vakcine proti HIVu, če tega nismo zmožni niti za vakcino proti SIVu, ki očitno ponuja preprost model.

Pod silnim pritiskom javnosti, zlasti pa nekaterih rizičnih skupin in inficiranih oseb, so zdravstvene oblasti ponekod nedavno dovolile uporabo nekaterih kandidatk-vakcin v preizkusne namene. Nacionalni inštitut za zdravje je začel študijo varnosti treh rekombinantnih vakcin na otrocih. Devetdeset s HIVom inficiranih otrok brez bolezenskih znamenj v starosti od 1 meseca do 12 let so s pristankom staršev izbrali in jih cepili s kandidatko-vakcino tvrdke Micro Gene System. Vakcino sestavlja virusni glikoprotein 120, ki je sestavina virusne ovojnice, in dve kandidatki-vakcini podobne sestave tvrdk Genentech in Biocine.

Podoben preizkus že opravljajo v armadi Združenih držav. Preizkušajo vakcino, ki jo sestavlja glikoprotein 160 tvrdke Micro Gene System. Ugotovili so, da s tem cepivom nekoliko povečajo količino navzočih protiteles, vendar vakcina ne varuje pred infekcijo. Strokovnjaki dvomijo v racionalnost tega preizkusa, kajti vakcino sestavlja glikoprotein 160, ki ni zguban, kot je naravni protein HIVa (13).

Težave so tudi s preizkusi kandidatke-vakcine na šimpanzih. HIV inficira šimpanze, vendar živali po infekciji ne zbolijo, pridelujejo pa protitelesa. Težava s temi preizkusi je, da so šimpanzi ena od živalskih posebej zavarovanih vrst, ker se je njihovo število v zadnjih letih silno zmanjšalo in jim grozi izumrtje.
Poskusi z vakcino proti HIVu se razločujejo od vseh podobnih preizkusov z vakcinami, kajti vakcinacija z antigeni HIVa pomeni lahko hudo diskriminacijo za vakcinirane osebe, kajti po cepljenju bodo serološko pozitivni. Nimamo pa še na voljo testov, s katerimi bi razločevali naravno od umetne infekcije s HIVom (14).

Vloga virusov pri raku

Štirideset let je minilo, kar smo javno govorili o vlogi virusov pri raku. Tistikrat ni bilo razumevanja za misel, da biološko ni sprejemljivo, da virusi sicer povzročajo tumorje in raka pri živalih, pri človeku pa ne. Danes vemo, da je približno 15% vseh rakov po svetu povezanih z virusno infekcijo.
Anogenitalne infekcije z virusi papiloma predstavljajo skoraj dve tretjini tega odstotka (15), v zadnji tretjini pa prevladujejo hepatokarcinomi, povezani z virusi hepatitisa, maligne bolezni, povezane z virusom Epstein-Barr in virusom humanega T limfotropnega virusa.
Danes tudi že vemo, da sama virusna infekcija ni dovolj za indukcijo tumorja. Pri humani anogenitalni infekciji s papilomavirusi nekaterih tipov so pridelki virusnih genov nujni, da se ohrani maligni fenotip. V nemalignih celicah, ki so latentno inficirane s papilomavirusi izražanje teh genov strogo nadzorujejo geni gostiteljeve celice, ki jih spodbujajo aktivirani makrofagi. Zdi se, da je neodzivnost genov gostiteljeve celice prvi pogoj za razvoj malignega fenotipa (16). Transdukcije, ki jih izzovejo persistentni virusni genomi, prispevajo pri nastanku tumorja v celicah, ki so inficirane z drugimi skupinami tumorskih virusov. Nekatere smo že omenili. Dodatno pa moramo računati še na učinke specifičnih integracijskih mest, ki tudi spremenijo vrsto proliferacije latentno inficiranih celic.
Opisani učinki so neposredni, virusi pa lahko prispevajo tudi s posrednimi učinki interakcije pri nastanku tumorja. Poti poznamo več. V zadnjem času je zlasti zanimiva imunska supresija, ki je značilna za infekcijo z virusom HIV. Druga pot je indukcija mutacij in amplifikacij (razmnoževanje v velikem številu) odlomkov DNA v celičnem genomu, kar se zgodi zlasti pogosto pri infekciji z virusi iz družine herpesvirusov. Ali še po tretji poti: z indukcijo amplifikacije in aktivacije z drugimi persistentnimi virusi, kar prav tako opazujemo pri herpesvirusih. Zanimivo je, da parvovirusi, odvisni od virusov pomočnikov, lahko delujejo nasprotno tem dogajanjem, ker zavirajo indukcijo mutacij in amplifikacij DNA. Spoznanje na videz neskladno z naravo virusov pa je nenavadno, kajti odpira nove možnosti za zdravljenje virusnih rakov v prihodnosti. Zamisel se bo marsikomu zdela preveč nenavadna, da bi bila uresničljiva, vendar so pogosto neznatna opazovanja pomenila prelomnico v razmišljanju in odpirale možnosti za zdravljenje ali spoznavanje bolezni.

Vpliv antivirusne terapije na klinično virologijo

Antivirusna terapija je postala v nekaterih primerih stvarnost. Zlasti uvajanje aciklo- in ganciklovirja je stvari docela spremenilo. Zdravljenja infekcij s herpesvirusi so pokazala, da je z antivirusnimi zdravili mogoče zmanjšati morbiditeto in mortaliteto herpesnega encefalitisa in herpesnih neonatalnih infekcij.
Uspehi so še večji, če omenimo še hitrejše zdravljenje infekcij z genitalnim herpesom in infekcij pri imunsko pomanjkljivih osebah. Uspešno zdravljenje herpesnih bolezni pa je le del učinka novih antivirusnih zdravil, kajti preprečevanje reaktivacije latentnih infekcij še mnogo bolj vpliva na preprečevanje herpesnih bolezni. Med uspehi naj navedemo supresijo reaktivacije genitalnega herpesa z aciklovirjem in supresijo citomegalije pri bolnikih po presajevanju organov ali tkiv z ganciklovirjem (17).
Ovrednotenje antiherpesne terapije v pravilno načrtovanih kliničnih preizkusih je bistveno povečalo tudi razumevanje patogeneze bolezni, kot sta neonatalna infekcija z virusom herpesa simpleksa in herpesvirusni encefalitis. Osvetlilo pa je tudi nevarnosti in tveganja, ki spremljajo te bolezni. Z uspešnim zdravljenjem herpesnih bolezni preprečujemo reaktivacijo letentne infekcije, kar še mnogo bolj vpliva na preprečevanje bolezni in infekcije. Dodatna pridobitev so še nove diagnostične metode za herpesni encefalitis, na primer z verižno reakcijo s polimerazo.
Zgodba o aciklo- in ganciklovirju je tisto, kar imenujemo »dobra novica«. Razprava o antivirusnih zdravilih pa še vedno ni pri kraju. Ko smo pred leti opravili predhodne teste z antiherpetikom, enim od derivatov tiazolidin ocetne kisline (18), smo naleteli na popolno nerazumevanje, zlasti farmakologov, ki so bili zaradi izkušenj v tistem času nadvse skeptični: »Virusne bolezni se razvijejo tako hitro, da zdravilo ne bo moglo delovati« ali pa: »Herpesne infekcije se pozdravi že z navadnim alkoholom«. Tudi stroški za razvijanje novega zdravila so tako veliki, da si jih lahko privoščijo le malokatere farmacevtske tvrdke po svetu.
Zdaj pa k »slabi novici«. V letošnji aprilski številki Lanceta je izšlo sporočilo skupine raziskovalcev projekta Concorde. Več skupin francoskih in angleških raziskovalcev je že leta 1988 začelo študijo, ali je dobrodejen učinek zivudina (azidotimidina, AZT) res takšen, kot poročajo raziskovalci tvrdke Wellcome, ki zdravilo izdeluje. Članek je sprožil veliko nasprotovanja, kajti s preprostimi besedami pove, da sicer jemanje AZT izboljša imunsko stanje oseb, ki so inficirane s HIVom, kar je očitno po zvečanju števila limfocitov T, vendar inficirane osebe zbolevajo in umirajo, kot bi zdravila sploh ne jemale. Tudi na IX. konferenci o aidsu v Berlinu letos so bile razprave o delovanju AZT nadvse vroče, raziskovalci projekta Concorde pa svojih trditev niso spremenili. Vprašanje je nadvse kritično, kajti zdravljenje posameznega bolnika z AZT stane na leto nekaj tisoč ameriških dolarjev.
Tvrdka Wellcome je od vsega začetka vložila veliko sredstev in tudi raziskovalnega dela, da so razvili AZT. Ko so sporočila raziskovalcev Concorde prišla v javnost, je padla vrednost delnic Wellcome za 500 milijonov funtov.

Literatura

1. Lwoff A. Introduction. In: Congress Int Virol. Ann Inst Past 1981; 132: 121–34.
2. Reyes GR, Purd MA, Kim JP, Luk KC, Young LM, Fry KE, Grdley DW. Isolation of cDNA from the viruses responsible for enterically transmitted nonA-nonB hepatitis. Science 1990; 247: 1335–9.
3. Bradley DW, Beach MJ, Purdy MA. Recent developments in the molecular cloning and characterization of hepatitis C and E virus. Mine review. Microbial Pathogenesis 1992; 12: 391–8.
4. Lipkin VI, Travis G, Carbone KM, Wilson MC. Isolation and characterization of Borna disease agent cDNA clones. Proc Natl Acad Sci 1990; 87: 4184–8.
5. Goebel SJ, Johnson GP, Perus ME, Davos SV, Winslow JP, Paoletti E. Appendix to the complete DNA sequence of vaccinia virus. Virol 1990; 179: 617–63.
6. Elliott RM, Crook NE, Desselberger U, Hull R, McGeoch DJ. Some highlights of virus research in 1990. J Gen Virol 1991; 72: 1761–79.
7. Becker Y, Darai D. Diagnosis of human viruses by polymerase chain reaction technology. Berlin: Springer Verlag, 1992; 1: 16–31.
8. Arnheiter H, Skuntz S, Notteborn M, Chang S, Meier E. Transgenic mice with intracellular immunity to influenza virus. Cell 1990; 62: 51–61.
9. Mulligan RC. Retroviral mediated gene transfer: basic and practical applications. Cong Virol Edinburgh 1993; Abstracts PL5–4.
10. Kolberg R. US alters cold virus to treat cystic fibrosis. New Sci 1992; Dec.: 12.
11. Schultz AM, Fast PE, Mathieson, Bradac J, Vogel FR, Walker MC. Status of AIDS vaccine development and testing. Berlin: IX Conf AIDS, 1993; Abstracts PO–A29.
12. Stott EJ, Mills KH, Page M, Taffs F, Cranage M, Greenway P, Kitchin P. Protection of cynomolguy macaques against simian immunodefficiency virus by fixed infected-cell vaccine. Lancet 1990; ii: 1538–41.
13. Sis C, Chong P, Mathews T, Bolognesi D, Klein M. Immunogenicity of linear and branched HIV-1 vaccine candidates. Berlin: IX Con AIDS, 1993; Abstracts: WS-A22-4.
14. Brown PH. Monkey puzzle and AIDS vaccines: no easy answers. Int Con AIFS Vaccine Development Florida 1992; Report 87–92.
15. Vandenvelde C, Beers van D. High risk genital papillomaviruses and degree of dysplastic changes in the cervix: A prospective study by fast multiplex polymerase chain reaction in Belgium. J Med Virol 1993; 39: 273–7.
16. Terry G, Ho L, Jenkins D, Mills M, Singer A, Mansell M, Beverly E. Definition of human papillomavirus type 16 DNA levels in low and high grade cervical lesions by a simple polymerase chain reaction technique. Arch Virol 1993; 128: 123–33.
17. Whitle RJ. Impact of antiviral therapy in clinical virology. Cong Virol 1993; Edinburgh Abstracts: PL5–5.
18. Likar M, Schauer P, Tišler M. An effective antiherpetic drug? Lancet 1968; 2: 776.

Professional article/Strokovni prispevek

HANTAVIRUS GENOMIC VARIATION AND DISEASE DISTRIBUTION

GENOMSKE VARIACIJE IN RAZŠIRJENOST HANTAVIRUSNIH INFEKCIJ V SVETU

Tatjana Avšič-Županc, Mario Poljak

Institute of Microbiology, Medical Faculty, Zaloška 4, 61105 Ljubljana, Slovenia

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Key words: *HFRS; hantaviruses; small mamals; PCR*

Abstract – Background. *Hemorrhagic fever with renal syndrome (HFRS) is a disease caused by viruses belonging to a genus Hantavirus of the family Bunyaviridae. Isolation of the Hantaan virus, the prototype virus of HFRS, in 1976 and its subsequent adaptation to grow in cell culture, quickly followed by development of an immunofluorescent antibody assay for serological diagnosis of infection, led to the conclusion that hantaviruses were widespread throughout the world in several genera of rodents and insectivores. Hantaviruses cause chronic, apparently asymptomatic infections among their reservoir hosts. Infection in man is a direct result of exposure to infected rodents or insectivores or their infected excreta.*

To date, at least four different serotypes (Hantaan, Puumala, Seoul, Prospect Hill) in the Hantavirus genus are registered and there is evidence of additional serotypes (Dobrava, Thailand, Thottapalayam). These viruses share antigenic, genetic, epidemiological and ecological characteristics. In general, milder form of HFRS is linked etiologically to Puumala serotype; severe and frequently fatal outcomes with Hantaan serotype, and generally milder, though potentially fatal, clinical variant with Seoul serotype.

Conclusions. *There is a considerable clinical as well as regional overlap, and geographic origin of the disease necessarily reveals a predictable pattern of illness. Moreover, there is emerging evidence that clinical patters as well as severity may vary by hantaviral serotypes within a given geographic region. To what extent hantavirus virulence variations and its different pathogenic consequences affect the human being is not yet known. Polymerase chain reaction (PCR) has been introduced as a method in studying variation and relatedness among hantavirus isolates at molecular level during recent years. Such analyses have proven to be very useful on classifying therefore, holding hantaviruses, great potential for future molecular epidemiological studies.*

Ključne besede: *HMRS; hantavirusi; majhni sesalci; PCR*

Izvleček – Izhodišča. *Hemoragična mrzlica z renalnim sindromom (HMRS) je akutna infekcija, ki jo povzročajo virusi iz rodu Hantavirus, družine Bunyaviridae. Osamitev prototipa hantavirusov, virusa Hantaan, leta 1976 in njegova prilagoditev za rast in razmnoževanje na kulturah celic je omogočila razvoj številnih seroloških metod za ugotavljanje okužbe s hantavirusi. Uporaba teh metod je privedla do spoznanja, da so hantavirusi široko razprostranjeni po vsem svetu v številnih rodovih glodalcev in žužkojedov. Hantavirusi povzročajo kronično, asimptomatsko okužbo svojih gostiteljev, predvsem prostoživečih majhnih sesalcev. Infekcija človeka je posledica neposredne izpostavitve okuženim glodalcem ali žužkojedom ter njihovim inficiranim izločkom.*

Znano je, da rod Hantavirus sestavljajo štirje različni serotipi: Hantaan, Puumala, Seoul in Prospect Hill. V novejšem času so dokazali še dodatne serotipe: Dobrava, Thailand, Thottapalayam. Različni hantavirusi imajo antigenske, genetične, epidemiološke in ekološke podobne značilnosti. Klinično je HMRS lahko izražena z različno težino bolezenskih znamenj. Navadno je blaga oblika bolezni etiološko vezana s Puumala hantavirusnim serotipom. Medtem ko je težka oblika bolezni, ki je pogostokrat smrtna, v povezavi s Hantaan serotipom, je Seoul serotip odgovoren za klinično zmerno težko obliko HMRS. Zaradi znatne povezave med klinično obliko bolezni in naravnim žariščem HMRS je mogoče na določenem zemljepisnem področju z veliko gotovostjo predvideti obliko bolezni, ki jo določen hantavirusni serotip povzroča. Predvsem zanimiva so področja, kjer je lahko navzočih več hantavirusnih serotipov hkrati.

Zaključki. *V novejšem času se metode molekularne biologije vse bolj uporabljajo za študij antigenskih inačic različnih virusov. Te metode, predvsem verižno reakcijo s polimerazo, smo vključili v naše raziskave. Rezultati molekularno epidemioloških raziskav hantavirusov v Sloveniji so pomemben prispevek k poznavanju hantavirusnih genomskih variacij in razširjenosti HMRS v svetu. Menimo, da bodo naši rezultati znatno pripomogli pri izbiri in preizkušanju sevov hantavirusov, primernih za izdelavo uspešnega cepiva, ki bi bil velik doprinos pri zatiranju te nevarne bolezni.*

Introduction

Hemorrhagic fever with renal syndrome (HFRS) refers to a number of human diseases with similar clinical symptoms characterized by fever and renal failure with or without hemorrhagic manifestations. The disease has been given a multitude of names, which contributed to the confusion concerning its actual distribution and epidemiology (1). In China, the disease is known as Epidemic hemorrhagic fever (EHF); in Korea, it is called Korean hemorrhagic fever (KHF); in Scandinavia, western part of former Soviet Union, and western Europe is called Nephropathia epidemica (NE). The World Health Organization adopted the term "hemorrhagic fever with renal syndrome" to serve as a unifying name for these and related conditions (1).

HFRS and hantaviruses

This group of diseases is caused by a newly recognized group of viruses, the genus Hantavirus, of the family Bunyaviridae (2). More than 100 hantavirus isolates have been made from human and rodent tissues obtained throughout the world. Systematic serological, molecular, and genetic studies have grouped them into at least 4 different serotypes: Hantaan, Seoul, Puumala, and Prospect Hill (3). Recently, evidence for additional serotypes was found (Dobrava, Thailand, Thottapalayam) (Tab. 1).

Tab. 1. *Members of the genus Hantavirus (Bunyaviridae), their rodent reservoirs, geographical distribution, and associated human disease.*

Virus	Primary rodent host	Distribution	Disease
Hantaan	Apodemus agrarius Apodemus flavicollis	China, Korea, USSR Balkans	EHF and KHF (severe) HFRS (severe)
Seoul	Rattus norvegicus	Worldwide	EHF (moderate)
Puumala	Clethrionomys glareolus	Scandinavia, USSR, Europe	Nephropathia epidemica
Prospect Hill	Microtus pennsylvanicus	USA (Maryland and Minnesota)	None known
Dobrava	Apodemus flavicollis	Slovenia	None known
Thailand	Bandicota indica	Thailand	None known
Thottapalayam	Suncus murinus	India	None known

Based on physical characteristics and genetic determinations, hantaviruses show the characteristic tripartite RNA genome of their unique 3' terminal sequence (4). The three segments, designated S, M and L segments, code for nucleocapside protein, envelope glycoproteins (G1 and G2) and a presumed transcriptase. Various strains of hantaviruses have been cloned and sequenced, and both surface glycoproteins and the core nucleocapside proteins have been expressed in different systems (3).

During recent years, polymerase chain reaction (PCR) has been introduced as a method to study variation and relatedness among various hantaviruses at molecular level. Both M and S segment PCR tests are currently being investigated, and universal primers that bind to amplify more than 30 strains of hantaviruses have been identified (5–7). After amplification, restriction endonucleases can be employed to differentiate isolates rapidly. Such analyses have been introduced also in our laboratory (8).

HFRS, hantaviruses and rodent hosts

Hantaviruses are maintened in nature primarily in rodents and insectivores (1). Hantaviruses cause chronic, apparently asymptomatic infections among their reservoir hosts. Infection in man is a direct result of an exposure to infected rodents or insectivores or their infected excreta. Rodents of the superfamily Muroidea, which includes the common species of rats, house mice, field and woodland mice and voles are the main reservoirs of hantaviruses. Although other mammals are occasionally infected with these viruses, in each geographic region where distinctive forms of HFRS occur, each hantavirus is primarily associated with a single species of rodent. Hantaan virus causes the most severe disease. Seoul virus produce disease of intermediate severity. Puumala virus causes the least severe disease. Prospect Hill virus does not appear to cause disease in humans (1).

HFRS is manifested clinically by fever, hypotension, hemorrhage and renal failure. It follows a complex course of illness with five recognized phases: febrile, hypotensive, oliguric, diuretic and convalescent (9).

It is clear from what has already been said that characteristics of the infecting virus are major determinants of the severity of illness. Hantaviruses and the disease they cause can best be understood by examining the individual viruses and the ecological relationship of their rodent hosts.

Korean hemorrhagic fever and Hantaan virus infection

What is now called "classic" HFRS due to Hantaan virus in Asia, is characterized by a severe disease progress in successive stages (10). The major clinical signs and symptoms seen in each stage are fever, shock, renal impairment, relative hypervolemia and fluid and electrolyte imbalance. A petechial rash may also be present, and a characteristic facial flushing develops in many patients. In patients with the more severe forms of the disease, hemorrhagic signs, including scleral injection, ecchymosis, and gastrointestinal bleeding, may be seen. In fatal cases, death is usually a consequence of shock or renal failure and occurs most frequently during the oliguric stage of disease (9). Recovery from infection was believed to be complete; however, reports of chronic renal impairment exist (1). Extensive surveys of potential animal reservoir in Korea failed to reveal additional hosts to Hantaan virus other than Apodemus agrarius, from which the prototype virus was isolated in 1976 by HW Lee in Korea (11).

Nephropathia epidemica and Puumala virus

A less severe form of HFRS, or Nephropathia epidemica, with case-mortality rate of less than 1% is found in Scandinavia, western part of former Soviet Union and other European countries (12). Although the disease spectrum of NE is similar to those of Far Eastern forms of HFRS, some of the clinical signs or symptoms are less prominent or occur in fever patients. Renal dysfunction is still the prominent clinical characteristic, but anuria is rare. Concentrating capacity of the kidney may be impaired from weeks to months, but serious hemorrhagic manifestation and mortality are generally absent. Puumala virus is the causative agent of NE and is maintained in nature by the bank vole Clethrionomys glareolus. The incidence of the illness in Scandinavia peaks in late fall and early winter, when voles invade manmade structures, and in mid-to-late summer, when people are active outdoors. The incidence is also affected by vole population cycles (12).

Seoul virus and urban rats

Seoul virus was first discovered by HW Lee and colleques during their investigations of Hantaan virus (13). They discovered that both black and roof rat, Rattus norvegicus and Rattus rattus are infected with Seoul virus and they spread the virus among urban residents. Seoul viruses produce a disease of moderate severity, in which hepatic involvement is prominent. After discovery of Seoul virus in urban rats in Korea, the possibility of potential dissemination of this zoonozis via shipping industry was suggested. Viruses similar or identical to Seoul virus were isolated from rats captured in the port cities of Philadelphia, Huston and Baltimore in USA (3). Serological surveys documented the presence of infected rats in many parts of Asia, Europe, Africa and South America. The infection of laboratory rat colonies and tissue lines derived from rats also pose a public health threat to certain occupational groups. Outbreaks of laboratory-rat associated HFRS have been documented in Korea, Japan, former Soviet Union, Belgium and United Kingdom (3). Generally, these infections are limited to animal handlers or laboratory personnel. Rutine screening of laboratory animals for viral pathogens now usually includes serological tests for hantaviruses (3).

Prospect Hill virus and Meadow voles

Prospect Hill virus was isolated from meadow voles Microtus pennsylvanicus captured in USA in the early 1980s (14). This virus is not currently considered a public health problem. Specific antibodies to this virus have been detected in sera collected from professional mammalogists (3). The infected individuals had no recollection of an illness compatible with HFRS.

Severe HFRS of the Balkans

Recently, an extremly severe type of HFRS was recognized in the mountain regions of Greece, Albania, Bulgaria, Serbia and Bosnia-Herzegovina (3, 15). The disease is far more severe than NE seen elsewhere in Europe and more closely resembles the Asian form of HFRS. Although relatively few cases have been recorded, the mortality rate appears to be even greater than in Asia. The origin of this severe forms of HFRS is currently not defined. The yellow-necked field mouse Apodemus flavicollis was found as the main reservoir of Hantaan serotype viruses (Porogia and Fojnica) at the Balkan Peninsula (3).

HFRS in Slovenia

The presence of HFRS in Slovenia was first reported in 1952 (16). Since then, 70 sporadic cases of HFRS have been documented in Slovenia. Both severe and mild cases are seen, with an overall mortality rate of 4.8% (17–20). Our serological and molecular epidemiological studies of HFRS in Slovenia indicated simultaneous circulation of different hantaviruses (8, 17–21). To date, three different hantaviruses were found in Slovenia. Hantaan virus was detected in A. flavicollis, field vole Microtus agrestis and frequently in dormouse Glis glis. Puumala virus was detected in C. glareolus only.

Dobrava virus – the third hantavirus found in Slovenia was isolated in 1988 from the lungs of an A. flavicollis captured in Dobrava village, a region where severe HFRS cases were discovered (22). Immunofluorescent antibody assays using convalescent human sera and MAbs indicate that Dobrava virus differs from all recognized hantaviruses (8). Enzyme immunoassays with rat immune sera specific for all serotypes of hantaviruses revealed that Dobrava virus is antigenically more related to Hantaan virus than to other serotypes (8, 19). Dobrava virus reacts more strongly with convalescent patients sera specific for Hantaan and Seoul viruses than with human sera specific for Puumala virus (23). Ideally, plaque reduction neutralization tests would definitively show the uniqueness of Dobrava virus; however, repeated attempts to plaque Dobrava virus have failed (19).

Recently, RNA from Dobrava virus infected cells was successfully reverse transcribed to cDNA and amplified by reverse transcriptase polymerase chain reaction (RT-PCR), and resultant amplimer from M-genomic segment was digested with restriction endonucleases (8). The restriction pattern was compared with those of the known hantaviruses and found to be clearly different. Finally, a portion of the M-genomic segment of Dobrava virus was amplified by PCR, and the nucleotide sequence determined (21). The comparison between the nucleotide sequence and the same gene region of other hantaviruses revealed an overall homology of 41.7%. A phylogenetic tree based on pair-wise sequence similarity clearly showed that Dobrava virus is genetically distinct, and probably represents a new virus in the genus Hantavirus of the family Bunyaviridae (21).

The hypothesis that characteristics of the infecting virus are major determinants of the severity of illness was confirmed also in Slovenia. In Prekmurje region, where C. glareolus was the predominant rodent species and Puumala virus the predominant hantavirus, a mild form of HFRS was mainly recognized. On the other hand, in Dolenjska region, where A. flavicollis and Hantaan virus predominated, a severe form of HFRS was frequent (18–20).

Conclusions

We have presented different types of hantaviruses which share antigenic, genetic, epidemiologic and ecologic characteristics. However, it remains to be determined how these antigenic differences are related to the pathogenic differences between these viruses.

Along with progress in our understanding of the epidemiology of the hantaviruses has come detailed molecular characterization of this new groups of viruses. Some of the molecular techniques, particularly PCR, have provided powerful new tools to aid in the diagnosis and epidemiological study of HFRS, and promise to provide a new vaccine candidate.

Results obtained from molecular epidemiological studies of hantaviruses in Slovenia are very important contribution to the knowledge of hantavirus genomic variation. We believe that our results represent a significant aid in selection of convenient hantavirus strain for vaccine preparation.

References

1. Lee HW, van der Groen G. Hemorrhagic fever with renal syndrome. Prog Med Virol Basel 1989; 36: 62–102.
2. White ID, Shirey FC, French GR et al. Hantaan virus, aetiological agent of Korean hemorrhagic fever, has Bunyaviridae-like morphology. Lancet 1982; 1: 768–71.
3. LeDuc JW, Childs JE, Glass GE. The hantaviruses, etiologic agents of hemorrhagic fever with renal syndrome: a possible cause of hypertension and chronic renal disease in the United States. Ann Rev Publ Health 1992; 13: 79–98.
4. Schmaljohn CS, Hasty SE, Dalrymple JM, LeDuc JW, Lee HW, Lee PW. Antigenic and genetic properties of viruses linked to hemorrhagic fever with renal syndrome. Science 1985; 227: 1041–4.
5. Xiao SY, Chu AK, Knauert FK et al. Comparison of hantavirus isolates using a genus-reactive primer pair polymerase chain reaction. J Gen Virol 1992; 73: 567–73.
6. Puthavathana P, Lee HW, Kang CY. Typing of hantaviruses from five continents by polymerase chain reaction. Virus Res 1992; 26: 1–14.
7. Arthur RR, Lofts RS, Gomez J et al. Grouping of Hantaviruses by small genome segment PCR and amplification of viral RNA from wild-caught rats. Am J Trop Med Hyg 1992; 47: 210–24.
8. Avšič-Županc T, Xiao SY, Stojanović R, Gligić A, van der Groen G, LeDuc JW. Characterization of Dobrava virus: a hantavirus from Slovenia. J Med Virol 1992; 38: 132–7.
9. Cosgriff TM. Hemorrhagic fever with renal syndrome: four decades of research. Ann Intern Med 1989; 110: 313–6.
10. Tsai TF. Hemorrhagic fever with renal syndrome: clinical aspects. Lab Anim Sci 1987; 37: 419–27.
11. Lee HW, Lee PW. Korean hemorrhagic fever. Demonstration of causative antigen and antibodies. Korean J Intern Med 1976; 19: 371–83.
12. Lahdevirta J. Nephropathia epidemica in Finland. A clinical, histological and epidemiological studies. Am Clin Res 1971; 3: Suppl 8: 1S–154S.
13. Lee HW, Beak LJ, Johnson KM. Isolation of Hantaan virus, the etiologic agent of Korean hemorrhagic fever, from wild urban rats. J Infect Dis 1982; 146: 638–44.
14. Lee PW, Amyx HL, Yanagihara R, Gajdusek CD, Goldgaber D, Gibbs CJ. Partial characterization of Prospect Hill virus isolated from meadow voles in the United States. J Infect Dis 1985; 152: 826–9.
15. Antoniadis A, LeDuc JW, Daniel-Alexiou S. Clinical and epidemiological aspects of hemorrhagic fever with renal syndrome in Greece. J Infect Dis 1987; 156: 295–301.
16. Radošević Z, Mohaček I. The problem of nephropathia epidemica Myhrman-Zetterholm in relation to acute interstitial nephritis. Acta Med Scand 1954; 149: 221–8.
17. Avšič-Županc T, Čižman G, Gligić A, van der Groen G. Evidence for hantavirus disease in Slovenia. Acta Virol 1989; 33: 327–37.
18. Avšič-Županc T, Likar M, Novaković S et al. Evidence of the presence of two hantaviruses in Slovenia. Arch Virol 1990; 115: Suppl 1: 87S–94S.
19. Avšič-Županc T. Antigenske lastnosti hantavirusa Dobrava in njegova patogenost. (Doktorska disertacija.) Ljubljana: Medicinska fakulteta, 1991.
20. Avšič-Županc T. Hemoragična mrzlica z renalnim sindromom v Sloveniji. Zdrav Vestn 1991; 60: 469–71.
21. Xiao SY, Diglišić G, Avšič-Županc T, LeDuc JW. Dobrava virus as a new hantavirus: evidenced by comparative sequence analysis. J Med Virol 1993; 39: 152–5.
22. Avšič-Županc T, Hoofd G, van der Groen G, Gligić A. Partial characterization of hantavirus isolated from Apodemus flavicollis captured in Slovenia. In: Proceedings of the First International Symposium on Hantaviruses and Crimen Congo hemorrhagic fever viruses. Thessaloniki: Aristotelian University, 1988; 50–2.
23. Groen J, Osterhaus ADME, Avšič-Županc T et al. Different hantavirus serotypes in western Europe. Lancet 1991; 337: 621–2.

Professional article/Strokovni prispevek

TYPHOID – MICROBIOLOGY AND PUBLIC HEALTH

TIFUS – MIKROBIOLOGIJA IN JAVNO ZDRAVSTVO

Branko Cvjetanović

School of Public Health "Andria Štampar", 41000 Zagreb, Croatia

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Key words: *Salmonella typhi; "intermittent carriers"; war and typhoid; stabilization; perspectives*

Abstract – Background. *In the introduction the author discusses the coordination between epidemiology and microbiology in general as well as in the eradication of typhoid as a case indicator. The role of "intermittent carriers" is presented in connection with the challenge dose of S. typhi. Mathematical models are seen as a method for the eradication of typhoid in global perspective. Mechanism of "self limitation" is described. Effects of war in the former Yugoslavia cannot be predicted but some data are given for Croatia. It is obvious that for the time being eradication of typhoid is interrupted, and some reasons are given: decline of personal and domestic hygiene, slackening of preventive measures, destruction of sanitary facilities and overtaxation of public health institutions.*

Conclusions. *Perspectives depend on the stability of the illness and the effects of war destruction. The author feels that, the future is uncertain.*

Ključne besede: *Salmonella typhi; "začasni bacilonosci"; vojna in tifus; stabilizacija; perspektive*

Izvleček – Izhodišča. *Članek v uvodu načenja vprašanje koordinacije med epidemiologijo in mikrobiologijo na splošno, nato pa preide na vprašanje trebušnega tifusa. Opozarja na vlogo "začasnih bacilonoscev". V matematičnih modelih vidi sredstvo za eradikacijo bolezni v globalnem obsegu, zlasti po mehanizmu "samoomejevanja". Prispevek je prva razprava pri nas o vlogi vojne na ozemlju bivše Jugoslavije in navaja podatke, ki so na voljo. Razumljivo je, da so razmere začasno ustavile eradikacijo trebušnega tifusa zaradi usihanja osebne higiene, opuščanja varstvenih ukrepov, uničenja infrastrukture in čezmerne obremenitve obstajajočih ustanov javnega zdravstva.*

Zaključki. *Napoved za prihodnost je odvisna od stabilizacije bolezni in učinkov uničenja sanitarnih ustanov in sredstev. Prihodnost se zdi avtorju negotova.*

Introduction

Microbiology and the banch work of microbiologist should, from the practical point of view, be seen in the light of their contribution to a sound public health strategy in the disease control. In the early days of this century, microbiologist and epidemiologist were one person, but with different techniques and specialities, it became necessary to coordinate their functions in a team work. It seems appropriate to consider the interrelation between these two disciplines in the combat of typhoid in an actual situation of the present ex-Yugoslavia.

While this presentation has a character of a "case study", an attempt will be made to bring up observations of wider significance for global aspects of typhoid control and its eventual eradication.

Disease dynamics

Salmonella typhi is one of the best studied microorganisms since it was isolated a century ago (1884). Characterization of the organism by sero- and phago-typing permitted epidemiological investigations into its spread, and thus establishment of effective control measures. The structure of the organisms is provided a basis for the development of the live vaccines. But some information of crucial importance for understanding of the mechanism and the dynamics of the infection came from what could be called "quantitative microbiology". Examination of the excreta of the chronic carriers indicated that there were no "intermittent carriers" but that the number of excreted bacteria varied and was often below the level detectable by current techniques (1). A very important finding of "quantitative microbiology" and experimental epidemiology was the determination of the challenge dose of Salmonella typhi to infect human volunteers (2). It has shown that the challenge dose is rather high (ID_{50} being between 10^6 and 10^7). However, in one study in the field, it appeared that the dose might be considerably lower (10^3) (3). From these studies and numerous observations it is apparent that infectivity of typhoid depends strictly on the challenge dose and the state of the host (immunity and susceptibility e. g. hypochlorhydria) and that in the developed countries with common standards of personal and communal sanitation and hygiene it is rather low.

Careful study of the natural history of typhoid and the construction of the mathematical model of its dynamics (4, 5) revealed that there is an intrinsic mechanism of self-limitation.

The "self-limiting mechanism" of typhoid fever is effective when the incidence of the disease by a gradual decrease passes the "threshold" of stable endemicity. At that point the dynamics of the population leads to the eventual eradication of the infection. This process obviously requires a stability of the population, living conditions and environment (4). Thus the mathematical model of the disease permits forcasting of typhoid dynamics. Validity of the model including "self-elimination mechanism" is proved by observations from the long and recent past dynamics and the trends of the disease in USA, UK and elsewhere (4, 6).

In practically all European countries the self-elimination process has began; this is not the case in the Middle East and the countries on the southern shores of the Mediterranean Sea (6).

Effects of the war on disease dynamics

In the northern parts of ex-Yugoslavia, as can be expected, the process of self-eradication of typhoid began long ago, while central and southern parts continued to have endemic typhoid. In Croatia, typhoid was in a steady decline.
In the last decade, the incidence of typhoid continued to decline (see table 1) and in 1991 reached the lowest number ever recorded. It seemed that the virtual eradication was approaching but then the war broke out and in the 1992 the recorded numer of cases returned to the level of ten years ago. This, of course, could have been expected; but what actually happened and what is to be expected?

Tab. 1. *Notified cases of typhoid.*

Croatia* – 1983–1992	
Year	No. of cases
1983	42
1984	18
1985	24
1986	9
1987	25
1988	19
1989	4
1990	6
1991	1
1992	42

* population 4.6 million

The war (as well as other disasters and calamities) means for the stricken areas and their inhabitants:
– decline of personal and domestic hygiene due to destruction of facilities,
– slackening of food safety; use of contaminated foods and unsafe water,
– destruction of sanitary infrastructure (water supply, sewage system, etc.),
– overtaxation of existing facilities, promiscuity in lodging and feeding, stress, harassment, casualties and deaths.
Apart from these generally untoward effects of war, some other specific unfavourable factors can add to the risk of transmission of typhoid, namely the influx of war refugees from endemic areas into areas of low incidence (and thus of high susceptibility).
All this indeed happened in Croatia.
The majority of cases (23) reported were among the refugees from Bosnia and Hercegovina where typhoid continued to be endemic, and who arrived to Croatia in the incubation period (7). During their retreat they were probably exposed to the most unsanitary conditions they ever experienced, and could not take usual precautions. Most of them followed the main stream of refugees leading them to Split (12 cases) and the vicinity (7 cases), but a few arrived to the north: Zagreb (2), Pula (1) and Daruvar (1). The majority of other cases were due to a waterborne outbreak (14 cases) which occured in heavily destructed city of Pakrac. These cases evidently resulted from destruction of the sanitary facilities by war; besides there were many refugees in the city and the vicinity that lived in temporary shelters. If these 14 cases are exempted, only 3 autochthonous cases occured (one in each city: Sisak and Čakovec in the north). One of them was a laboratory infection in Split.
It is obvious that the natural epidemiological process of self-elimination of typhoid in Croatia continued, and that the increased number of cases is a direct consequence of the war.

Prospectives

The future will depend on the result of the two opposite forces:
a) a stabilizing, self-elimination process and
b) untoward effects of war; destruction of sanitary facilities, migration etc.

a) The first means a stability of the population, a functioning of sanitary facilities and of the public health services.

b) The war brings, apart from destruction of sanitary structure, refugees and migration, also poverty, because of which many people may become destitutes and thus be forced to abandon their healthy life style, and ultimately become victims of typhoid and other infections.
Another danger of international concern is a possible spill-over from Bosnia and Hercegovina via Croatia or directly to other countries. Therefore, all should benefit if the war is stopped, even those who declare that their interests are not at stake.

What should be done?

The seriousness of the situation is fully appreciated by those concerned. "Those concerned" in this particular case are health services of Croatia, both civilian and military, and they continue with the surveillance system, trying to improve it, since epidemiological information is a basis for an effective public health action.
As to the future of typhoid in this part of the world, several things are certain, in spite of a number of uncertainties. War will end one day and the reconstruction, including sanitary facilities, will return the conditions to their earlier level and beyond, and typhoid, after its rise during the war, will continue on its road of self-eradication. Encouraging observation of spectacular drop of typhoid in Japan after World War Two shows that it can happen. Whether it will be so in this part of the world, or the recovery will be slower, is uncertain; but it will come.

Discussion

This opens the questions what should be global public health strategy and what is to be the role of microbiology and microbiologists.
Should the strategy be typhoid control or its eradication? Since there is the self-limiting intrinsic mechanism in this infection why not eradicate it?
Obviously not with a world-wide, billions of dollars worth spectacular campaign, but by steady and well-planned work first to bring all countries to the threshold of a stable endemicity and then let the self-eradication mechanism work by supporting it through rational use of available resources.
I quote one of the most eminent students of typhoid dr. R.B. Hornick (8): "Improvement in sanitation has a predictable and long-lasting beneficial effect. All countries need to address this problem so that typhoid and other enteric infections can be eradicated." It seems that "International Task Force for Disease Eradication (ITFDE)" is going to consider this possibility.
Be as it may, typhoid will be with us and because of the severity of the disease, it will be necessary to deal with it. Medical microbiologists will have to continue with the diagnosis of the sick and the detection of the carriers, but environmental microbiologists will have an important role in tracing modes of spread through food, water and environment and to guide sanitarians and ecologists in their work aimed at typhoid and other enteric infection control and eradication. As to the epidemiologists who will collaborate with "environmental microbiologists", the question arises whether they should, as public health agents, be attached to the ministry of environment where the resources could be obtained by environmental interventions.
Perhaps the time has come to change the strategy and the tasks of microbiologists and public health workers in the typhoid control with the aim of eradication of the disease.

References

1. Mersalis JG Jr., Kaye D, Connelly CS, Hook EW. Quantitative bacteriology of the typhoid carrier state. Amer J Trop Med 1964; 13: 425–9.
2. Hornick RB, Woodward TE. Apprisal of typhoid vaccine in experimentally infected human subjects. Trans Clin Climat Assoc 1967; 78: 70–8.
3. Cvjetanović B. Controlled field trial of alcohol and phenol antityphoid vaccines. In Typhoid Commission: Effectiveness of Anti-typhoid Immunization. Centr Hig Zavod, Zagreb; p. 25–6 (in Croatian).
4. Cvjetanović B, Grab B, Uemura K. Dynamics of Acute Bacterial Diseases, Epidemiological models and their application in public health. Wld Hlt Org Bulletin 1978; vol. 56, Suppl 1, Chapter 4: Typhoid, 45–64.
5. Cvjetanović B, Grab B, Dixon H. Computerized epidemiological model of typhoid fever with age structure and its use in the planning and evaluation of anti-typhoid immunization and sanitation programmes. Mathematical Modelling 1986; 7: 719–44.
6. Cvjetanović B. Typhoid and its control in the Mediterranean. In Tourist Health, Proceedings of the second international conference on tourist health, Rimini, March, 1989.
7. Epidemiološki Vjesnik (Epidemiological Records), Zavod za zaštitu zdravlja rep. Hrvatske (Institute of Health Protection of Croatia) – Odjel za Epidemiologiju (Dpt. of Epidemiol.), 1992; 12.
8. Hornick RB. Selective Primary Health Care: Strategies for control of disease in the developing world. Typhoid fever; Reviews of Infectious Diseases, 1985; XX, 7: 536–46.
9. Morbidity and Mortality Weekly Report. US Centres for Disease Control, 1992; 41: 37.

Review article/Pregledni prispevek

INTERFERONS IN ANTITUMOR THERAPY: CURRENT STATUS AND FUTURE PROSPECTS

INTERFERONI PRI ZDRAVLJENJU TUMORJEV: STANJE DANES IN IZGLEDI ZA PRIHODNOST

W. Robert Fleischmann, Jr.

Department of Microbiology and Immunology, The University of Texas Medical Branch, Galveston, TX 77555, USA

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Key words: *interferons; antitumor activity; clinical use*

Ključne besede: *interferoni; protitumorska dejavnost; klinična uporaba*

Abstract – Background. *Interferons are currently approved for approximately 15 clinical applications in a number of different countries. However, while interferons do have many clinical uses, they also cause significant side effects in treated individuals. Furthermore, the in vivo activities of the interferons against many tumors are many-fold lower than expected on the basis of in vitro observations. A major challenge for the future is to increase the therapeutic index of the interferons by reducing their side effects and by increasing their antitumor activities. A number of possible methods for increasing the therapeutic index of interferons are currently under investigation.*

Conclusions. *Early results of animal and in vitro studies have been promising. However, these studies must be extended to the human system before their true utility can be measured. Based upon the high biological significance of the studies performed to date in animal models, it is likely that interferons have not yet reached their maximal potential.*

Izvleček – Izhodišča. *Interferoni so registrirani v številnih državah kot klinično učinkovite snovi za zdravljenje različnih virusnih in rakavih bolezni. Njihovo uporabnost ovirajo številni nezaželeni stranski učinki. Poleg tega je dejavnost interferonov in vivo mnogo manjša, kot bi pričakovali na osnovi poskusov, opravljenih in vitro. Posebej pomembno se zdi poiskati načine, kako povečati terapevtski indeks interferonov oz. kako povečati protitumorsko učinkovitost ob hkratnem zmanjševanju stranskih učinkov.*

Zaključki. *Laboratorijske in vitro raziskave in poskusi na živalskih modelih so nakazali nekaj obetavnih možnosti za dosego tega cilja, dobljene rezultate pa je treba preveriti v kliniki. Vsekakor mnoge biološko signifikantne predklinične študije zbujajo upanje, da bo v bodočnosti mogoče izboljšati klinično učinkovitost in uporabnost interferonov.*

Introduction

Biological response modifiers are natural cell products which have broad activities both in vitro and in vivo. Interferons, the topic of this review, are biological response modifiers. Three major types of interferons have been identified in man (for review, see 1). These interferons have been identified by the first three letters of the Greek alphabet as IFN-α, IFN-β, and IFN-γ.

General Characteristics and Biological Activities of Interferons

As shown in Table 1, different interferons are produced by different kinds of cells (for review, see 1). IFN-α is produced primarily by leukocytes of the macrophage and B lymphocyte lineages. IFN-β is produced by epithelial and fibroblast cells. IFN-γ is produced primarily by leukocytes of the T-cell and natural killer cell lineages. Interferons can be induced by a number of endogenous as well as exogenous inducers. The primary endogenous inducers of interferons are other members of the biological response modifier family. Indeed, the complex interactions of the various biological response modifiers are currently being defined.

A number of exogenous inducers have been identified. IFN-α is induced by the exposure of producing cells to viruses, B-cell mitogens, or tumors. IFN-β is induced by exposure of producing cells to viruses or foreign nucleic acids. IFN–γ is induced by exposure of producing cells to foreign antigens and T-cell mitogens.

Tab. 1. *Types of human interferons, their producing cells, and inducers.*

Types of interferons	Producing cells	Inducers
IFN-α	Macrophages/dendritic cells	Viruses
	B lymphocytes	B cell mitogens
	Null lymphocytes	Tumors
IFN-β	Epithelial cells	Viruses
	Fibroblast cells	Foreign nucleic acids
IFN–γ	CD4 T helper-1 cells	Foreign antigens
	CD8 cytotoxic suppressor cells	T-cell mitogens
	Natural killer cells	

Despite the differences in the producing cells and the inducing stimuli, IFN-α and IFN-β are related molecules (for review, see 2–3). As shown in Table 2, the genes for IFN-α and IFN-β are located in adjacent regions of chromosome 9. The interferon genes for IFN-α encode 13 highly related proteins composed of 166 amino acids that differ by as little as a single amino acid. The interferon

gene for IFN-β shares about 50% homology at the nucleotide level with the IFN-α genes and encodes a single protein composed of 166 amino acids. Further, IFN-α and IFN-β activate treated cells via the same cell surface receptor and induce an identical set of approximately 12 proteins in treated cells.

Tab. 2. *Distinguishing characteristics of human interferons by type.*

Characteristic	IFN-α	IFN-β	IFN–γ
Genes			
Numer	13	1	1
Chromosomal location	Chromosome 9	Chromosome 9	Chromosome 12
Introns	0	0	3
Number of amino acids	166	166	146
Glycosylation	No	Yes	Yes
Acid Stability	Yes/No	Yes	No
Receptor	Same as for IFN-β	Same as for IFN-α	Distinct
Activity			
Synergic interaction	With IFN–γ	With IFN–γ	With IFN-α&IFN-β
Polypeptides induces	~12	~12	~24
Host range	Wide	Restricted	Restricted

In contrast, the gene for IFN–γ is located on chromosome 12, is not believed to be related to IFN-α and IFN-β, and encodes a protein of 146 amino acids (for review, see 4). IFN–γ activates treated cells via a cell surface receptor that is distinct from the receptor for IFN-α and IFN-β. Finally, IFN–γ induces approximately 24 proteins in treated cells, some of which overlap those proteins induced by IFN-α and IFN-β.

Interferons were first recognized for their antiviral activity more than 35 years ago. Over the years, interferons have been shown to have a broad range of additional activities, including regulation of cell growth and differentiation, immunoregulation, activation of cytotoxic immune cells, hormone-like activity, and antitumor activity (Tab. 3). It should be noted that while the three interferons differ in their general characteristics, they all exhibit these same broad biological activities, albeit to variable extents.

Tab. 3. *Activities of human interferons.*

Antiviral action
Cell growth inhibition
Cellular differentiation
Immunoregulatory actions
Activation of cytotoxic cells
Hormonal interactions
Antitumor actions

Antitumor Actions of Interferons

Studies showing that interferons regulate cell growth and differentiation and have profound effects on the host immune system led to the consideration that interferons might have antitumor activity. Indeed, in vitro and animal studies have confirmed that interferons exert potent antitumor effects. The antitumor effects of interferons are mediated through a wide variety of mechanisms. As shown in Table 4, these antitumor effects can be subdivided into direct and indirect antitumor effects.

Direct antitumor effects are those that result from direct interaction of the interferons with the tumor cells. For example, treatment of tumor cells with IFN–γ can result in the lysis of the tumor cells. Other effects are less dramatic, but no less effective, and include decreased polyamine and tryptophan biosynthesis, induction of 2'5' oligoadenylate synthetase, decreased oncogene expression, and increased expression of specific cell surface molecules.

Indirect antitumor effects are those that result from activation of cells of the host immune system, which then, either directly or indirectly, exert the antitumor effect. For example, interferon treatment can activate cytotoxic effector cells of the host immune system to kill specifically tumor cells. Interferons can also modulate antibody production, decreasing the amount of protective antibodies that may interfere with the action of the cytotoxic effector cells. Finally, interferons can stimulate the production of a cascade of other cytokines and lymphokines that may have cytotoxic (tumor necrosis factor) or growth inhibiting activity (IFN-α or IFN-β production following treatment with IFN–γ) (for review, see 5).

Tab. 4. *Antitumor activities of human interferons.*

Direct antitumor effects	Indirect antitumor effects
Antiproliferative effects	Activation of cytotoxic effector cells
Decrease in polyamine biosynthesis	Activation of macrophages/monocytes
Decrease in tryptophan biosynthesis	Activation of T cells
Induction of 2'5' oligoadenylate synthetase	Activation of NK cells
Modulation of proto-oncogene expression	
	Modulation of antibody production
Cytotoxic effects	
Direct lysis	Induction of other lymphokines and cytokines
Enhancement of cell surface antigens	
Increase in class I antigen expression	
Increase in class II antigen expression	
Increase in tumor-associated antigens	
Enhancement of TNF receptors	

Clinical Use of Interferons

The preclinical observations cited above have supported a clinical role for interferon in the management of viral, immunoregulatory, and benign as well as malignant neoplastic diseases (for review, see 6). As summarized in Table 5, IFN-α is currently employed in a number of countries to control chronic diseases associated with hepatitis B and hepatitis C viral infections. IFN-β is employed to treat multiple sclerosis, a disease of the central nervous system that is believed to have both viral and immunological components. IFN–γ is employed to treat chronic granulomatous disease. In addition, IFN-β is being used to treat cervical intraepithelial neoplasia, and IFN-α has been licensed to treat a wide variety of benign and malignant neoplasias.

Tab. 5. *Approved applications of human interferons worldwide.*[1]

Disease	Number of countries	Interferon type
Hairy cell leukemia	32	IFN-α
Kaposi's sarcoma in AIDS	22	IFN-α
Chronic hepatitis B	19	IFN-α
Chronic hepatitis C	10	IFN-α
Condyloma acuminatum	10	IFN-α
Mycosis fungoides	10	IFN-α
Chronic myeloid leukemia	8	IFN-α
Renal cell carcinoma	8	IFN-α
Malignant melanoma	5	IFN-α
Multiple myeloma	5	IFN-α
Juvenile laryngeal papilloma	5	IFN-α
Non-Hodgkin's lymphoma	2	IFN-α
Malignant carcinoid tumor	1	IFN-α
Chronic granulomatous disease	1	IFN–γ
Cervical intraepithelial neoplasia	1	IFN-β
Multiple sclerosis	1	IFN-β

[1] Adapted from reference 6.

While interferons have been shown to have dramatic effects in the treatment of some conditions such as hairy cell leukemia (greater than 80-90% response rate; 5% complete remission rate) (for review, see 7), chronic myelogenous leukemia (73% complete hematologic remissions) (for review, see 7), condyloma acuminatum (greater than 50% complete remission rate) (Stephen Tyring, personal communication), and juvenile laryngeal papilloma (50–80% response rate) (for review, see 8), they have not been equally impressive in the treatment of most of the other diseases and conditions. For example, the response rate for interferon treatment of malignant melanoma is only about 10–15% with even fewer

complete responses (for review, see 8). Thus, there is certainly room for improvement of interferons' clinical activity.
Another consideration is that the clinical use of interferons is associated with both acute and chronic side effects (for review, see 9). As shown in Table 6, acute side effects commonly include fever and chills, malaise, myalgias, and mild neutropenia. Chronic side effects commonly include fatigue, anorexia, mild neutropenia, transaminase elevations, diarrhea, and depression.

Tab. 6. *Clinical side effects of human interferon.*

Acute	Chronic
Common:	Fatigue
Chills	Anorexia
Fever	Mild neutropenia
Malaise	Transaminase elevations
Myalgias	Diarrhea
Mild neutropenia	Depression
Less common:	
Mental slowing	
Confusion	
Hair shedding	
Thrombocytopenia	
Nausea	
Vomiting	

Methods for Improving Interferon Therapy

Because of the limited antitumor effect of interferons against most tumors and because of the significant side effects associated with interferon therapy, methods to improve the therapeutic index of interferons are needed. Table 7 outlines a number of possible methods that could be used to improve interferon therapy.

Tab. 7. *Possible methods for improving human interferon therapy.*

Optimization of Therapy
– determination of the ideal level of interferon dosage, – determination of the ideal dosage interval, – determination of the most appropriate interferon to employ, – determination of the most appropriate chronobiologic treatment time.
Combination Therapy
– employment of synergistic combinations of interferons, – employment of interferons in combination with other lymphokines and cytokines (i. e., IL-2, TNF, etc.), – employment of interferons in combination with hyperthermia, – employment of interferons in combination with chemotherapy, – employment of interferons before surgery.
In Vitro Interferon Treatments
– treatment of bone marrow from leukemics with interferon before reinjection, – treatment of tumor cells obtained from biopsies with interferon; reinjection of dead tumor cells.

Optimization of therapy

One method to optimize therapy would be to determine the ideal level of interferon dosage to be used for different tumors. The effect of dosage level as it relates to specific types of tumors has not been well-studied to date. It might be anticipated that the tumors which are more responsive to the direct effects of interferons might respond well to relatively higher interferon doses, while those more responsive to the indirect effects of interferons might respond better to relatively lower interferon doses that more efficiently activate the host immune system.
Similarity, the ideal dosage interval is yet to be determined. The recommended initial therapy means administering IFN-α for five successive days for several weeks, followed by maintenance treatment of three doses per week. It has been shown in in vitro studies that treatment with IFN-α for four successive days can result in physiological resistance to the interferon (10). If this resistance occurs in vivo, it might be best to treat with IFN-α on alternate days rather than every day. Furthermore, in vitro studies have shown that treatment with IFN–γ can reverse resistance to IFN-α, which suggests that alternating treatments with IFN-α and IFN–γ might be more effective than prolonged treatment with IFN-α alone (11).
Different tumor cells respond differentially to the three types of interferon (12). It will be important to determine for each type of tumor and perhaps for each tumor (by in vitro sensitivity studies on biopsy material), the interferon that is most effective against that tumor.
Another method to optimize therapy would be to determine the most appropriate chronobiologic treatment time. Studies in the mouse system have shown that the therapeutic index of IFN-α and IFN–γ can be dramatically increased by choosing the most appropriate time for interferon treatment (13). For example, treatment of mice with IFN–γ at 16 hours after light onset (in a 12 hours of light, 12 hours of dark regimen) can reduce the bone marrow suppressive effects of the interferon by about 18-fold and increase the antitumor effect by at least 9-fold, resulting in an increase in the therapeutic index of IFN–γ of about 160-fold. If similar chronobiological effects are seen in man, the clinical usefulness of interferons may be increased significantly.

Combination therapy

Combinations of IFN–γ with either IFN-α or IFN-β have been shown, in animal and in vitro studies, to give a synergistically enhanced antitumor action (for review, see 14). Similarly, other combinations of lymphokines and cytokines have been shown to interact synergistically in preclinical studies. Some clinical studies have confirmed these synergistic interactions, but many have not. More studies must be performed to determine the best combinations of lymphokines and cytokines and the best ratios of different lymphokines and cytokines in various combinations for these possibilities to be evaluated adequately.
Animal and tissue culture studies have shown that modest hyperthermia (2°C rise in whole body temperature) can synergistically enhance the antitumor activity of IFN–γ (12, 15). Parallel studies have shown that combined treatment with interferons and cytotoxic chemotherapeutic drugs can give an enhanced antitumor action (16).
Following surgery, a suppression of the immune system is often seen. Interferon treatment before and after surgery might be of general use in preventing the immunosuppression and of specific use in boosting the immune system to attack metastatic sites not removed by surgery.

In vitro interferon treatments

Studies in mice have recently shown that in vitro treatment of B16 melanoma with IFN-α can be used to activate the host immune system (17). In the experiments, B16 melanoma cells were treated with interferon in vitro, killed, and injected into mice bearing untreated B16 melanoma tumors. The immune systems of the tumor-bearing mice were in some measure activated by this treatment to attack the B16 melanoma tumors, resulting in significant increases in survival time. These studies suggest that interferon treatment of bone marrow cultures taken from leukemics might be treated with interferon in vitro, killed, and reinjected into the patient's, possibly resulting in the activation of the patient immune system to attack the remaining leukemic cells. Similarly, biopsied primary tumor cells (obtained during surgery to remove the primary tumor) might be cultured with interferon in vitro, killed, and reinjected into the patient.
While the proposed methods for improving interferon therapy discussed above are speculative, the studies in animals and in vitro have given encouraging results. Of course, much more work needs to be done before their true potential can be accurately evaluated.

However, the preclinical studies suggest that some of these methods for enhancing interferon therapy may indeed be useful in the clinical treatment of human tumors.

Conclusions

Interferons have been employed clinically for the treatment of a number of viral, benign, and malignant disease conditions. However, the efficacy of interferons against most diseases is still low and must be improved. A number of methods for the improvement of the interferon efficacy are currently under evaluation in preclinical studies. These studies have shown some potential and may lead to a more efficacious clinical use of interferons.

References

1. Baron S, Coppenhaver DH, Dianzani F et al. Introduction to the interferon system. In: Interferon: Principles and Medical Applications. Baron S, Coppenhaver DH, Dianzani F et al. eds. Galveston, TX: University of Texas Medical Branch Press, 1992: 1–15.
2. Zoon KC, Bekisz J, Miller D. Human interferon alpha family: protein structure and function. In: Interferon: Principles and Medical Applications. Baron S, Coppenhaver DH, Dianzani F et al. eds. Galveston, TX: University of Texas Medical Branch Press, 1992: 95–105.
3. Utsumi J, Shimizu H. Human interferon β, protein structure and function. In: Interferon: Principles and Medical Applications. Baron S, Coppenhaver DH, Dianzani F et al. eds. Galveston, TX: University of Texas Medical Branch Press, 1992: 107–27.
4. Trotta PP, Nagabhushan TL. Gamma interferon: protein structure and function. In: Interferon: Principles and Medical Applications. Baron S, Coppenhaver DH, Dianzani F et al. eds. Galveston, TX: University of Texas Medical Branch Press, 1992: 129–38.
5. Baron S, Tyring SK, Fleischmann WR Jr. et al. Mechanisms of interferon action and clinical applications. JAMA 1991; 266: 1375–81.
6. Tyring SK. Introduction to clinical uses of interferons. In: Interferon: Principles and Medical Applications. Baron S, Coppenhaver DH, Dianzani F et al. eds. Galveston, TX: University of Texas Medical Branch Press, 1992: 399–407.
7. Platanias LC, Golomb HM. Clinical use of interferons: hairy cell, chronic myelogenous, and other leukemias. In: Interferon: Principles and Medical Applications. Baron S, Coppenhaver DH, Dianzani F et al. eds. Galveston, TX: University of Texas Medical Branch Press, 1992: 477–99.
8. Strander H, Oberg K. Clinical use of interferons: solid tumors. In: Interferon: Principles and Medical Applications. Baron S, Coppenhaver DH, Dianzani F et al. eds. Galveston, TX: University of Texas Medical Branch Press, 1992: 533–61.
9. Quesada JR. Toxicity and side effects of interferons. In: Interferon: Principles and Medical Applications. Baron S, Coppenhaver DH, Dianzani F et al. eds. Galveston, TX: University of Texas Medical Branch Press, 1992: 427–32.
10. Fleischmann CM, Fleischmann WR Jr. Differential antiproliferative activities of IFNs α, β, and γ: kinetics of establishment of their antiproliferative effects and the rapid development of resistance to IFNsα and β. J Biol Regul Homeost Agents 1988; 2: 173–85.
11. Fleischmann CM, Fleischmann WR Jr. Resistance to the antiproliferative activity of IFN-α: further characterization and demonstration of antagonistic effects of IFN–γ. J Biol Regul Homeost Agents 1991; 5: 34–42.
12. Fleischmann CM, Fleischmann WR Jr. Effects of hyperthermia on the in vitro antiproliferative activities of HuIFN-alpha, HuIFN-beta, and HuIFN-gamma employed separately and in combination. J Biol Regul Homeost Agents 1988; 2: 145–54.
13. Koren S, Whorton EB, Fleischmann WR Jr. Circadian dependence of interferon antitumor activity in mice. J Natl Cancer Inst 1993; 85: 1927–32.
14. Fleischmann WR Jr., Koren S. The use of combination therapies to enhance interferon action. In: The Interferon System: A Current Review to 1987. Baron S, Dianzani F, Stanton GJ, and Fleischmann WR Jr. eds. Galveston, TX: University of Texas Medical Branch Press, 1987: 241–52.
15. Anjum A, Fleischmann WR Jr. Effect of hyperthermia on the antitumor actions of interferons. J Biol Regul Homeost Agents 1992; 6: 75–86.
16. Namba M, Yamamoto S, Tanaka H, Kanamori T, Nobuhara M, Kimoto T. In vitro and in vivo studies on potentiation of cytotoxic of anticancer drugs or cobalt 60 gamma ray by interferon in human neoplastic cells. Cancer 1984; 54: 2262–7.
17. Fleischmann CM. Tumor studies using tumor cells resistant to interferon. This volume.

Review article/Pregledni prispevek

THE ROLE OF INTERFERONS IN THE REGULATION OF CELL FUNCTION AND IN THE PATHOGENESIS OF DISEASE

VLOGA INTERFERONOV PRI URAVNAVANJU DELOVANJA CELIC IN PATOGENEZI BOLEZNI

Renata Mažuran

Institute of Immunology, Rockefellerova 10, 41000 Zagreb, Croatia

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Key words: *interferons; cytokines; pathogenesis; therapy*

Abstract – Background. *Interferons are integral and efficient cell communication and regulation molecules. They are implicated in homeostasis and defense against many diseases and are involved in the pathogenesis of a variety of disorders. As a result of their regulatory potential, interferons are used as therapeutic agents in cancer, infectious diseases, inflammatory processes and autoimmune disorders. One example of this pervasiveness is the potential use of interferons in cancer treatment; where they are being tested as direct antitumor agents, as enhancers of immunological reactivity against tumor cells and as potentiators of chemotherapy and radiotherapy.*

Conclusions. *The purpose of this presentation is to describe new findings in interferon biology, particularly those answering the questions how the interferons are regulated, how their effects on cells are mediated, and how they interact with other cytokines in complex regulatory networks. Finally, it shows how such information can be used to understand and influence normal cell function and disease pathogenesis.*

Ključne besede: *interferoni; citokini; patogeneza; zdravljenje*

Izvleček – Izhodišča. *V številnih laboratorijskih poskusih in študijah je bilo nedvomno prikazano, da interferoni zavirajo rast primarnih tumorjev in preprečujejo razvoj metastaz. Poleg tega se uporabljajo v kombinaciji s kemoterapijo in radioterapijo kot uspešna dodatna oblika zdravljenja z majhno stopnjo toksičnosti. Za optimalno uporabo interferonov je potrebno poleg natančnega poznavanja protivirusnega in protitumorskega učinkovanja še nadrobno razumevanje njihovega delovanja v fizioloških pogojih. Na tem področju je bil dosežen ogromen napredek. Ko so uspeli z genetskim inženirstvom izdelati rekombinantne interferone, je bilo mogoče preučevati zgradbo in delovanje interferonov na molekulski ravni in jih pridobiti v dovolj velikih količinah, potrebnih za obsežne klinične raziskave.*

Zaključki. *Interferoni so regulacijske molekule, ki delujejo v kompleksni mreži citokinov. Vzdržujejo homeostazo in uravnavajo nespecifične in specifične obrambne mehanizme, vpleteni pa so tudi v patogenezo mnogih bolezni. Znano je, da je pri mnogih bolezenskih stanjih (kronični hepatitis B, levkemije, AIDS) znižana raven endogenega interferona. Zaradi njihovega regulatornega delovanja uporabljajo interferone v terapiji nalezljivih bolezni, tumorjev, vnetnih procesov in avtoimunih bolezni. Zaključiti je mogoče, da je za smiselno in ciljno uporabo interferonov potrebno boljše poznavanje fiziološke proizvodnje in dejavnosti citokinov, vključno z interferoni in natančnejše razumevanje interakcij znotraj citokinske mreže.*

Introduction

This review is focused on some past, present and potential roles of interferons as therapeutic agents. The article was partly drawn up on behalf of the Institute of Immunology, Zagreb, as a tribute to the efforts of its associates (producers, scientists, clinicians) which have been carried on for the last two decades in the field of interferon biology.

The Institute of Immunology in Zagreb started the production of human leukocyte interferon in 1970. It has been more than 22 years since we first presented the data, at the International Symposium on tumors of the head and neck in Zadar (1971) and at the 12th International Congress for Microbiological Standardization in Annecy (1971), which show that a natural protein, nonpurified crude human leukocyte interferon, could prevent or even cure viral infections and benign neoplasms. In Croatia, interferon was licensed as a preparation for human use in 1972. The initial results were from trials with locally administered interferon as it is shown in Tab. 1.

During the period from 1975 to 1989, we tested the preparations of human natural leukocyte interferon in controlled clinical trials (Tab. 2). Thanks to the clinical researches, human leukocyte interferon from our institute is now systematically administered to the patients with different pathological conditions.

In addition, we published numerous studies to evaluate the possible mechanisms of action of human leukocyte interferon in vitro and in vivo. The results are presented in Tab. 3.

The results of our research generally confirm those published by others (20). It means that apart from effecting the primary tumors, which were significantly reduced, interferons can clearly inhibit the development of tumor metastases (2, 3, 6). They are even more effective in combination with other agents (chemotherapy, radio-

therapy) (8, 11) than alone. They are potentially good adjuvants because, in small doses, they prolong the resistance to the disease after primary onset. We have also observed a "priming effect" on the constitutively produced interferon. We did not notice any serious side effects during prolonged therapeutic regimens. Most common are fever and pain, erythema and induration at the site of i.m. injection if a nonpurified preparation is used.

Criteria for therapeutic use of interferons

To use interferons optimally we must understand how they act on normal as well as on diseased tissues. Better understanding of the variables that govern interferon action and at least partial identification of the mechanisms leading to the beneficial effect in various disorders will lead to successful clinical trials. Since the early 1960s, there was enormous progress on this field. There are, however, still some clinicians and scientists who define interferons as "a group of new, expensive biological agents, which is entering routine clinical practice in selected applications" (21).
In 1980s advances in biotechnology were applied to IFN production; in 1979 interferon, interferon-alpha1 to be precise, was the first cloned cytokine (22). The benefit was more than unlimited supply of fully purified interferon; the direct result of cloning of interferon was characterization of interferons on molecular basis (Tab. 4).

What are the principles that we have learned by using this drug?

The therapeutic effects do not improve with the higher doses. As experience with recombinant interferons proved, the side effects were the same as those caused by crude interferons. They were dose-related and became almost intolerable when more than 20 MU were given, even for relatively short periods of time. Many trials were initiated on treating both viral infections and malignancies with "sufficiently high" doses of interferon; the results have been disappointing. Obviously, the complex nature of interferons as biological system was still not given enough concern.

What are the "targets" for interferon therapy in future?

Interferons are an integral part of the cytokine network and we have to accept that they, as other cytokines, may be produced constitutively at low levels. How we can use this endogenous system as a therapeutic agent? Simply by keeping following postulates in our mind:
1. Cytokines are hormone-like compounds with effects on the cell of origin (autocrine effect), cells in the microenvironment (paracrine effect), and distant cells (endocrine effect). Cytokines mediate cell-to-cell communication; interferons share the ability to induce, stimulate, and repress distinct gene expression with other members of cytokine family. They are multifunctional molecules (redundancy) that can regulate a wide spectrum of biologic events (pleiotropy) relevant to inflammation, metabolism, cell growth and differentiation, morphogenesis, fibrogenesis, and/or homeostasis.
2. The term "cytokine network" (24) indicates that the biological function of several cytokines overlaps and that two or more cytokines can antagonize each other or may have synergistic effects. Furthermore, the production and the release of cytokines are influenced by positive and negative feedback loops from the same and other cytokines. In fact, cellular homeostasis probably represents a balance between the effects caused by a variety of cytokines which together constitute cytokine network.

Tab. 1. *Local application of human leukocyte interferon. During the four five-year period 1970–1975, 539 patients were treated with frequent, small doses of interferon (10^3 to 10^4 IU) locally administered in a form of eye-drops (oil suspension), ointment, freeze-dried product or s.c. injections. Each clinical trial was double-blind and included controls treated either with placebo, or conventional therapy. The results were promising, with high percentage of completely cured lesions on skin or mucosa (1).*

Viral disease of	
– eye	epidemic keratoconjunctivitis, vaccinal blepharoconjunctivitis and keratitis caused by vaccinia virus;
– skin	herpes zoster, treatment and prevention of postvaccinal reactions after mass vaccination against smallpox;
– mucous membranes	
mouth	gingivostomatitis, recurrent labial herpes;
nose	influenza A;
genito-anal region	genital herpes;
Benign tumors	condylomata accuminata, verruca cornua, vulgaris and plana;
Malignant tumors	cancer of the vulva, planocellular cervical cancer, basalioma

Tab. 2. *Controlled clinical trials with natural human leukocyte interferon (Institute of Immunology, Zagreb, Croatia) during the period 1975–1989. Interferon was administered by intramuscular injections ($2–3\times10^6$ IU daily), s.c. injections, suppositoria ($0.5–1\times10^6$ IU), vaginaletes (10^6 IU), intrapleurally by instillation of thin needle, or through Omaya reservoir.*

Skin, head and neck tumors	Padovan I et al., 1979 (2) Ikić D et al., 1981 (3)
Uterine cervix precancerosis	Ikić D et al., 1981 (4)
Squamous cell carcinoma of uterine cervix	Ikić D et al., 1981 (5)
Urinary bladder papillomatosis, breast cancer, melanoma	Ikić D et al., 1981 (6)
Virus B hepatitis	Vlatković et al., 1986 (7)
Breast cancer with pleural carcinomatosis	Jereb B et al., 1987 (8) Mažuran R et al., 1992 (9)
Juvenile laryngeal papillomatosis	Krajina Z et al., 1989 (10)
Malignant brain tumors	Jereb B et al., 1989 (11)
Cervical intraepithelial neoplasia	Singer Z et al., 1990 (12)
Non-small cell lung cancer	Terčelj-Zorman M et al., 1991 (13)

Additionally, the biological effects of cytokines are often situation specific since they vary depending not only on the concentration of the cytokine or the presence or absence of other cytokines in the local microenvironment but also on the state of activation and/or maturation of the target cell and the composition and state of degradation of the surrounding matrix. It is only with an appreciation of this context that the biologic role of a cytokine can truly be determined (25).
3. Physiological function of cytokines has been difficult to determine despite their plethora of activities in vitro. The perfect example showing how deep interferons are involved in the development of immune reaction, preferentially of delayed type, is described below:
Over the past year or so, three cytokines (Il-2, Il-4, and Il-10) have been subjected to the ultimate test through deletion of their genes in mice by targeting techniques. Studies now indicate that deleting the gene for Il-2 does have important effects on the immune system even in young mice. The principal disturbance is on the balance of lymphokines produced by $CD4^+$ T cells, which regulate one another's production. Recently, at least three distinct cytokine secretion patterns have been defined among both murine and human $CD4^+$ T cells. Il-2 and interferon-gamma are mainly produced by Th 1 clones whereas Il-4, Il-5, Il-6 and Il-10 are principally produced by Th2 cells. Th0 cells exhibit a mixed pattern of cytokine production. In the Il-2-mice, some of the "Th2 type" lymphokines were overproduced upon in vitro stimulation, and serum titres of IgG1 and IgE, both of which are regulated by Il-4, were strikingly increased (26). The addition of interferon-

Tab. 3. *"The tip of the iceberg": immunomodulation by human leukocyte interferon in vitro on human mononuclear cells in short-term cultures and in vivo in peripheral blood of healthy volunteers and patients with different diseases.*

In vitro effect on:	
monocyte/macrophage	
– inhibition of spreading	Mažuran R et al., 1983 (14)
natural killer cells	Ikić M et al., 1983 (15)
cultivated tumor cells	
– invasive properties	Ikić D et al., 1982 (16)
– proliferation	
In vivo effect on:	
– serum interferon levels	Mažuran R et al., 1983 (17) Mažuran R et al., 1987 (18) Mažuran R et al., 1992 (9)
– serum cortisol levels	Mažuran R et al., 1987 (18)
– antibody production	Vodopija J et al., 1981 (19)
– natural killer cell activity	Mažuran R et al., 1983 (17) Mažuran R et al., 1987 (18)
– ADCC, phagocytosis, lymphocyte proliferation	Mažuran R et al., 1983 (17)
– chronobiological rhythms of serum IFN levels and NK-cell activity	Mažuran R et., 1992 (9)

Tab. 4. *Characterization of interferons (IFNs) (modified from [23]).*

Type	IFN-alpha[1] (leukocyte)	IFN-beta[2] (fibroblast)	IFN-gamma ("immune")
Produced by	peripheral leukocytes	fibroblasts	lymphocytes
Inducing agent	virus infection, dsRNA	virus infection, dsRNA	mitogens, specific antigens
Number of genes	at least 15 functional genes and 9 pseudogenes	1	1
Chromosomal location	9	9	12
Size of actual protein (No. of amino acids)	143	145	146
Glycoprotein	No	Yes	Yes

[1] Another IFN-alpha exists, primarily in the sera of patients with autoimmune disease, but also among IFNs produced by virus-induced leukocytes, which is antigenically distinct from all other interferons, acid-labile, and about 60% related genetically to IFNs-alpha and about 26% to IFN-beta. This IFN previously referred to as IFN-alpha$_1$ or omega, is now designated as IFN-alpha$_{10}$, and the classical group of alpha-interferons is now designated as IFN-alpha$_1$.

[2] When induced, many cells produce a second glycoprotein, in addition to IFN-beta, which cross-reacts with antisera against IFN-beta and was therefore named IFN-beta 2. However, the gene that encodes this protein is located on chromosome 7 and possesses little sequence similarity with IFN-beta: encodes a protein which is 184 amino acids long and the antiviral activity which is far lower than that of other IFNs-alpha and beta. It is, however, a very powerful cytokine, named Il-6.

gamma induced those T cells to differentiate into Th0, or even Th1 instead into Th2 clones. The interferon-alpha produced predominantly by macrophages, that present the antigen to Th cells also promotes such differentiation. Therefore, it is reasonable to suggest that, given the capacity of viruses and intracellular bacteria to stimulate macrophage production of interferon-alpha and Il-2 (which induce interferon-gamma production by both T cells and NK cells), as well as to activate $CD8^+$ T cells (which produce high amounts of interferon-gamma), $CD4^+$ Th cells may be simultaneously presented with processed antigen plus cytokines that induce them to differentiate towards a Th1 phenotype. In other words, viruses and intracellular bacteria induce Th1 responses because the profile of the "natural" immune response they evoke provides optimum conditions (high concentrations of interferon-gamma and absence of Il-4).

4. Endogenous interferon deficiencies seem to play a role in different diseases, including chronic hepatitis B, leukemia (interferon genes were deleted in number of leukemia cell lines), and AIDS. The postulate that interferons as therapeutic agents seem to be active in many diseases irrespective of their role in pathogenesis somehow sounds illogical. The recent paper by Génot et al. (27) proved how deeply interferon-alpha is involved in pathogenesis and therapy. Namely, they described the mechanism by which interferon-alpha downregulates the abnormal intracytoplasmic free calcium concentration of tumor cells in hairy cell leukemia, a disease with exquisite sensitivity to interferon-alpha therapy. Hairy cells display a higher Ca^{2+} than normal resting or anti-μ-activated B cells which might be due to aberrant control of Ca^{2+} fluxes leading to Ca^{2+} accumulation into leukemic cells. A sustained enhancement of free intracytoplasmic Ca^{2+} may prevent a further differentiation of hairy cells. Ligation of interferon-alpha to its receptor provides a signal necessary for dephosphorylation of CD20 molecule (B-cell specific phosphoprotein involved in controlling cell cycle progression). The in vivo correlation between the diminution of CD20 phosphorylation and Ca^{2+} in tumor cells samples from patients at the beginning of interferon-alpha therapy suggests that these two parameters are connected.

Conclusion

Therapeutic interventions that alter the production and/or effector function of cytokines are now receiving widespread attention.

In diseases that result from over-exuberant cytokine effector function, therapeutically useful interventions will be needed to obviate the pathologic effect of the cytokines without negating the beneficial effects that result from physiologic cytokine production. Sites of cytokine-cytokine synergy are attractive targets for these anticytokine interventions, because the effects that result from these interactions can be ameliorated by the neutralization of only one of the interacting molecules or the partial blockade of both molecules.

In diseases that result from a cytokine defficiency or respond to an increase in cytokine activity, therapeutically useful interventions will provide the necessary effector molecules without producing significant cytotoxicity. A knowledge of cytokine-cytokine synergy will also help in these situations.

References

1. Ikić D ed. Proceedings of 9th international Symposium on Clinical use of Interferon. Zagreb: Yugoslav Academy of Sciences and Arts, 1975.
2. Padovan I, Brodarec I, Ikić D, Knežević M, Šooš E. Effect of interferon in therapy of head and neck tumors. In: Khan A, Hill NO, Dorn GL eds. Interferon: Properties and clinical uses. Dallas: Leland Fikes Foundation Press, 1979: 713–22.
3. Ikić D, Padovan I, Brodarec I, Knežević M, Šooš E. Application of human leukocyte interferon in patients with tumors of the head and neck. Lancet 1981; 1: 1025–7.
4. Ikić D, Singer Z, Beck M, Šooš E, Šips D, Jušić D. Interferon treatment of uterine cervical precancerosis. J Cancer Res Clin Oncol 1981; 101: 303–8.
5. Ikić D, Krušić J, Kirhmajer V et al. Application of human leukocyte interferon in patients with carcinoma of the uterine cervix. Lancet 1981; 1: 1027–30.
6. Ikić D, Nola P, Maričić Z et al. Application of human leukocyte interferon in patients with urinary bladder papillomatosis, breast cancer and melanoma. Lancet 1981; 1: 1022–4.
7. Vlatković R, Jušić D, Šooš E, Miling M, Gvojić B, Iskra M. Human leukocyte interferon in therapy of hepatitis B virus infections. In: Filipič B ed. Yugoslave colloquium on IFN, Slovenian Microbiological Society. Ljubljana, 1986: 99–104.
8. Jereb B, Us-Krašovec M, Cervek J, Šooš E. Intrapleural application of human leukocyte interferon (HLI) in breast cancer patients with ipsilateral pleural carcinomatosis. J Interferon Res 1987; 7: 357–63.
9. Mažuran R, Ikić-Sutlić M, Jereb B et al. Intrapleural application of natural IFN alpha in breast cancer patients with pleural carcinomatosis. Monitoring of immunotherapy by assaying serum interferon levels. J Biol Regul Homeostat Agents 1992; 6: 46–52.
10. Krajina Z, Lenarčić-Čepelja I, Jušić D, Mažuran R, Vranešić D, Stavljenić A. Juvenile laryngeal papillomatosis. Liječ Vijesn 1989; 111: 419–23.
11. Jereb B, Petrič J, Lamovec J, Škrbec M, Šooš E. Intratumor application of human leukocyte interferon-alpha in patients with malignant brain tumors. Am J Clin Oncol (CCT) 1989; 12: 1–7.
12. Singer Z, Beck M, Jušić D, Šooš E. Treatment of cervical intraepithelial neoplasia (CIN) with HPV infections by interferon vaginalettes. J Exp Clin Cancer Res 1990; Suppl; abstracts of 7th World Congress of Cervical Pathology and Colposcopy, Rome; 190.
13. Terčelj-Zorman M, Mermolja M, Jereb B et al. Human leukocyte interferon alpha (HLI-alpha) for treatment of pleural effusion caused by non small lung cancer. Acta Oncol 1991; 30: 963–5.

14. Mažuran R, Sabioncello A, Rajninger-Miholić M, Jušić D, Šooš E. Negative correlation between the antiviral titre of a human leukocyte interferon and its ability to inhibit monocyte spreading in vitro. Period Biol 1983; 85: Suppl 3: 169–70.
15. Ikić M, Mažuran R, Šooš E, Silobrčić V. Effect of human leukocyte interferons on in vitro cytotoxicity of natural killer cells. Period Biol 1983; 85: Suppl 3: 171–1.
16. Ikić D, Beck M, Juzbašić M et al. Antiviral and antiinvasive titers of human interferons. Int J Clin Pharmacol Ther Toxicol 1982; 20: 179–80.
17. Mažuran R, Jušić D, Rabatić S et al. Effect of a single dose of crude leukocyte interferon on natural immunity in healthy volunteers. Proceedings of international meeting on Clinical and Biological Evaluation of Immunomodulators. Como, 1983: 26–6.
18. Mažuran R, Delač J, Jušić D, Tomašić J, Valinger Z, Ikić-Sutlić M. Interrelationship between serum cortisol and NK-activity during interferon therapy. Period Biol 1987; 89: Suppl 1: 163–3.
19. Vodopija J, Smerdel S, Baklaić Ž, Svjetličić M, Ljubičić M. Treatment of persons exposed to rabies virus with human diploid cell strain rabies vaccine and human leukocyte interferon. In: De Meyer E, Galasso G, Shellekens H eds. The biology of the interferon system. Amsterdam: Elsevier/North Holland Biomedical Press, 1981: 371–4.
20. Gresser I. Antitumor effects of interferons: past, present and future. Br J Haematol 1991; 79: 1–5.
21. Stuart-Harris RC, Lauchlan R, Day R. The clinical application of the interferons. Med J Aust 1992; 156: 869–72.
22. Weissmann C. The cloning of interferon and other mistakes. In: Gresser I ed. Interferon 1981. Vol. 3. London: Academic Press, 1981: 101–34.
23. Joklik WK. Interferons. In: Fields BN, Knipe DM eds. Virology. New York: Raven Press Ltd., 1990: 383–410.
24. Balkwill FR, Burke F. The cytokine network. Immunology Today 1989; 10: 299–303.
25. Elias JA, Zitnik RJ. Cytokine-cytokine interactions in the context of cytokine networking. Am J Resp Cell Mol Biol 1992; 7: 365–7.
26. Paul WE. Cytokines: Poking holes in the network. Nature 1992; 357: 16–7.
27. Génot E, Bismuth G, Degos L, Sigaux F, Wietzerbin J. Interferon-alpha downregulates the abnormal intracytoplasmic free calcium concentration of tumor cells in hairy cell leukemia. Blood 1992; 80: 2060–5.

Professional article/Strokovni prispevek

TUMOR STUDIES USING TUMOR CELLS RESISTANT TO INTERFERON

ODPORNOST TUMORSKIH CELIC ZA INTERFERON

Christina M. Fleischmann

Department of Microbiology and Immunology, The University of Texas Medical Branch, Galveston, TX 77555, USA

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Key words: *interferon; tumor cells; resistance*

Ključne besede: *interferon; tumorske celice; odpornost*

Abstract – Background. *An understanding of the contributions of the various IFN-induced antitumor effects would be beneficial for decision making regarding the clinical use of the IFN.*

Methods. *Physiologically IFN-resistant B16 melanoma cells (B16α^{res} cells) were studied in an in vivo tumor model.*

Results. *B16 melanoma cells resistant to the in vitro antiproliferative effects of IFN were more sensitive to the in vivo antitumor effects of the same IFN.*

Conclusion. *The B16α^{res} cells are useful for the study of IFN-induced host-mediated antitumor effects and appear to be significantly more immunogenic than the B16 cells.*

Izvleček – Izhodišča. *Z natančnejšim razumevanjem neposrednih in posrednih protitumorskih dejavnosti interferona in mehanizmov odpornosti tumorskih celic za interferon bi lahko dosegli smotrnejšo in učinkovitejšo uporabo interferonov v kliniki.*

Metode. *Na interferon alfa odporne B16 melanomske celice so bile preučevane v in vivo tumorskem modelu.*

Rezultati. *B16 melanomske celice, odporne na antiproliferativno delovanje interferona in vitro, so bile v primerjavi z neodpornimi B16 celicami bolj občutljive na protitumorske učinke interferona in vivo.*

Zaključek. *B16 melanomske celice, fiziološko odporne na interferon alfa, so koristne za preučevanje protitumorskega delovanja interferonov. Zdi se, da so odporne B 16 celice signifikantno bolj imunogene kot neodporne B 16 celice.*

Introduction

Interferons (IFNs) are a group of naturally produced proteins which have antiviral, antitumor, and immunomodulatory effects. Antitumor effects of IFNs include those directed at the tumor cell (direct effects) and those directed at the activation of host immune defenses (indirect effects) (reviewed in 1). An understanding of the interplay of the direct and indirect antitumor effects is important to obtain the optimal clinical effect from IFNs. It has proven difficult, however, to separate the direct effects from the indirect effects.

The indirect antitumor effects of IFN have been studied with human tumor explants in nude mice and with IFN-resistant tumor cells, because these systems exclude direct IFN-mediated effects on the tumor cells themselves. Most IFN-resistant tumor studies have used resistant cell clones selected, from long-term culture in increasing concentrations of IFN-α (2–13). The resulting IFN-resistant clones are highly selected, genetically resistant and maintain the resistant phenotype for months or years after removal from IFN. In general, IFN antitumor studies using these systems have shown that the resistant cell tumors are about as sensitive to the indirect antitumor effects of the IFN as the IFN-sensitive parental cells.

In contrast to the work with genetically stable resistant cell clones, previous work in this laboratory has involved physiologically resistant tumor cells. Murine B16 melanoma cells and human G361 malignant melanoma cells have been shown to become rapidly resistant to the direct antiproliferative effects of IFN-α and IFN-β in vitro (14, 15). This resistance is generalized, nongenetic, and dose-dependent according to the concentration of IFN to which the cells are exposed. The resistant B16 melanoma cells (B16α^{res} cells) remain sensitive to the antiviral effects of IFN-α and to the antiviral and antiproliferative effects of IFN-γ.

The current research has involved the study of the resistance in an in vivo mouse tumor model system. The possible in vivo development of resistance to the antitumor effects of IFN-α was investigated. In addition, B16α^{res} cells were used in the in vivo tumor model system.

Methods

Cells and IFN

Mouse B16 melanoma cells (clone F-1) (16) were grown in 100 mm plastic tissue culture dishes (Corning, Corning, NY) in a growth medium of Eagle's minimal essential medium (Earle's base EMEM, GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (FCS, JRH Biologicals, Lenexa, KS) and antibiotics. In vitro resistant cells (B16α^{res} cells) were generated by in vitro growth in 10^4 U/ml IFN-α prior to inoculation into the mice. Recombinant human IFN-α, rHuIFN-α A/D (IFN-α), donated by M. Brunda (Hoffman-LaRoche, Nutley, NJ), has a specific activity of $10^{7.81}$ units/mg of protein.

Mice

Pathogen-free female C57Bl/6 mice were obtained from Jackson Laboratory (Bar Harbor, ME). They were maintained in a pathogen-free state as determined by antibody testing.

Intraperitoneal tumor model

C57Bl/6 mice were intraperitoneally (i. p.) injected with either B16 or B16α^{res} cells resuspended in EMEM + 2% FCS at a concentration of 10^6 cells unless otherwise stated. Mice were then assigned to groups and were treated either with phosphate buffered saline containing 3 mg/ml bovine serum albumin (PBS/BSA) or with PBS/BSA containing 10^4 U/injection of IFN-α. The treatment began 24 h after the tumor inoculation and continued daily for either 5 or 19 days. Mice were monitored for day of death. The average length of survival was calculated for each group, and the Increased Life Span (ILS) was calculated for each IFN-treated mouse using the formula:

$$ILS = \frac{\text{Day of death for IFN-treated mouse} - \text{Average day of death for control mouse}}{\text{Average day of death for control mice}}$$

Results

B16 melanoma cells rapidly become resistant to the in vitro antiproliferative effects of IFN-α. It was possible that resistance to the antitumor effects of IFN-α might develop also in vivo. To test this, B16 melanoma cells were injected i.p. into C57Bl/6 mice. Some mice received a 5-day IFN-α treatment, while others received a 19-day IFN-α treatment. Control groups received PBS/BSA only. The results presented in Tab. 1 show that the mice that received the 19-day IFN-α treatment survived significantly longer than the mice that received the 5-day IFN-α treatment ($p = 0.001$). Thus, it appeared that in vivo development of resistance, if it occured in our tumor model, did not shorten survival times of the treated mice.

Tab. 1. *Survival of mice treated with IFN-α for 5 or 19 days following B16 cell inoculation.*

Length of treatment	Number of mice	Day of death	ILS
0 days	34	16.7	
5 days	34	27.9	67
19 days	21	37.2	123

Next, B16α^{res} cells were compared to B16 cells in the in vivo tumor model. The mice received a 5-day IFN-α treatment or control treatment following inoculation of either B16α^{res} or B16 cells. The data summarized in Tab. 2 indicate, surprisingly, that IFN-treated B16α^{res}-inoculated mice had a significantly increased ILS compared to IFN-treated B16-inoculated mice ($p = 0.013$). Apparently, B16α^{res} cells are more sensitive to the IFN-induced host-mediated antitumor effects.

Tab. 2. *Survival comparisons for IFN-treated mice inoculated with either B16 or B16α^{res} cells.*

Cells	Treatment	Number of mice	Day of death	ILS
B16	PBS/BSA	41	16.5	
	IFN-α	41	27.8	69
B16α^{res}	PBS/BSA	51	20.8	
	IFN-α	54	42.1	102

Cure rates are also important indicators of the antitumor effectiveness of IFN-α. Therefore, the cure rates of the IFN-treated B16-inoculated mice and the IFN-treated B16α^{res}-inoculated mice were compared. The cumulative results are presented in Tab. 3. When treated with IFN-α for 19 days, 29% of the mice inoculated with B16 cells and 70% of the mice inoculated with B16α^{res} survived tumor free for 90 days. This difference was significant as determined by chi square analysis ($p = 0.0019$). Therefore, IFN-treated B16α^{res}-inoculated mice had a substantially higher cure rate as well as a substantially higher ILS.

Tab. 3. *Cure rate comparisons for IFN-treated mice inoculated with either B16 or B16α^{res} cells.*

Cells	Survivors/Mice inoculated	Survival (%)	Comparison
B16	11/38	29	
B16α^{res}	16/23	70	$p = 0.0019$

Discussion

In order to understand the complex antitumor effects induced by IFN, it is useful to study model systems in which the direct and the indirect effects may be separated. The use of in vitro IFN-resistant tumor cells allows the study of IFN-induced indirect antitumor effects in the absence of a direct IFN antitumor effect. This tumor model system uses physiologically resistant tumor cells rather than genetically resistant tumor cells. As such, it may be a more relevant model system than those previously employed.

The results for this tumor model system show that resistance to the direct in vivo antitumor effects of IFN-α does not appear to shorten survival of IFN-treated mice. In addition, when IFN-treated mice had been inoculated with in vitro resistant B16α^{res} cells, their average survival was significantly enhanced compared to the average survival of similarly treated B16-inoculated mice. Finally, the 90-day cure rate of the IFN-treated B16α^{res}-inoculated mice was significantly higher than the 90-day cure rate of IFN-treated B16-inoculated mice. It is possible that the in vitro resistant B16α^{res} melanoma cells were more immunogenic than the B16 melanoma cells due to the in vitro culturing in the presence of IFN-α. These B16α^{res} cells appeared to elicit an enhanced immune response in IFN-treated mice. This tumor model system might be useful for future studies of IFN-induced antitumor effects.

References

1. Fleischmann WR Jr., Fleischmann CM. Mechanisms of interferons' antitumor actions. In: Baron S, Coppenhaver D, Dianzani F et al. eds. Interferon: Principles and medical applications. Galveston: The University of Texas Medical Branch Press, 1992: 299–310.
2. Belardelli F, Gresser I, Maury C, Maunoury M-T. Antitumor effects of interferon in mice injected with interferon-sensitive and interferon-resistant Friend leukemia cells. I. Int J Cancer 1982; 30: 813–20.
3. Belardelli F, Gresser I, Maury C, Maunoury M-T. Antitumor effects of interferon in mice injected with interferon-sensitive and interferon-resistant Friend leukemia cells. II. Role of host mechanisms. Int J Cancer 1982; 30: 821–5.
4. Lee S, Chiu H, Rinderknecht E, Sabo W, Stebbing N. Importance of treatment regimen of interferon as an antitumor agent. Cancer Res 1983; 43: 4172–5.
5. Brunda M, Rosenbaum D, Stern L. Inhibition of experimentally-induced murine metastases by recombinant alpha interferon: correlation between the modulatory effect of interferon treatment on natural killer cell activity and inhibition of metastases. Int J Cancer 1984; 34: 421–6.
6. Nishimura J, Mitsui K, Ishikawa T et al. Antitumor and antimetastatic activities of human recombinant interferon alpha A/D. Clin Expl Metastasis 1985; 3: 295–304.
7. Uno K, Shimizu S, Ido M et al. Direct and indirect effects of interferon on in vivo murine tumor cell growth. Cancer Res 1985; 45: 1320–7.
8. Uno K, Shimizu S, Inaba K et al. Effect of recombinant human interferon-α A/D on in vivo murine tumor cell growth. Cancer Res 1988; 48: 2366–71.
9. Maekawa R, Kitagawa T, Hojo K, Wada T, Sato K. Distinct antitumor mechanisms of recombinant murine interferon-γ against two murine tumor models. J. Interferon Res 1988; 8: 227–39.
10. Tabata Y, Uno K, Muramatsu S, Ikada Y. In vivo effects of recombinant interferon alpha A/D incorporated in gelatin microspheres on murine tumor cell growth. Jpn J Cancer Res 1989; 80: 387–93.
11. Reid TR, Race ER, Wolff BH, Friedman RM, Merigan TC, Basham TY. Enhanced in vivo therapeutic response to interferon in mice with an in vitro interferon-resistant B-cell lymphoma. Cancer Res 1989; 49: 4163–9.
12. Gresser I, Maury C, Carnaud C, De Maeyer E, Maunoury M-T, Belardelli F. Antitumor effects of interferon in mice injected with interferon-sensitive and interferon-resistant Friend erythroleukemia cells. VIII. Role of the immune system in the inhibition of visceral metastases. Int J Cancer 1990; 46: 468–74.

13. Gresser I. Antitumor effects of interferons: past, present and future. Brit J Haematol 1991; 79: Suppl 1: 1–5.
14. Fleischmann CM, Fleischmann WR Jr. Differential antiproliferative activities of IFNs α, β and γ: Kinetics of establishment of their antiproliferative effects and the rapid development of resistance to IFNs α and β. J Biol Regul Homeost Agents 1988; 2: 173–85.
15. Fleischmann CM, Fleischmann WR Jr. Resistance to the antiproliferative activity of IFN-α: further characterization and demonstration of antagonistic effects of IFN-γ. J Biol Regul Homeost Agents 1991; 5: 34–42.
16. Fidler IJ. Selection of successive tumor lines for metastasis. Nature New Biol 1973; 242: 148–9.

Professional article/Strokovni prispevek

CHRONOBIOLOGY OF THE INTERFERON SYSTEM

KRONOBIOLOGIJA INTERFERONSKEGA SISTEMA

Srečko Koren[1], W. R. Fleischmann Jr.[2]

[1] Institute of Microbiology, Medical Faculty, Zaloška 4, 61105 Ljubljana, Slovenia
[2] Department of Microbiology and Immunology, The University of Texas Medical Branch, Galveston, TX 77555, USA

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Key words: *interferons; chronobiology; circadian rhythmicity; antitumor activity; myelosuppressive activity*

Abstract – *The interferon (IFN) system consists of three different IFN types (alpha, beta, and gamma) which operate through a coordinate series of cellular events leading to antiviral, antitumor and immunomodulatory responses. Sporadic data from clinical studies have suggested that there may be a time-dependent variation in the severity of the most common toxic side effects of IFNs in man. More detailed chronobiological studies in a murine experimental model have revealed that various activities of IFNs rhythmically change during the circadian period of approximatelly 24 hours. Circadian rhythmicity in antitumor as well as myelosuppressive activity of IFN-alpha and IFN-gamma was discovered and characterized.*

The time points at which IFN-alpha exerted its maximum and minimum antitumor effect were different from those observed for IFN-gamma. In an analogous way, the time points of maximum and minimum myelosuppressive effect were different for the two IFN types. The knowledge of predictable rhythmic changes in the susceptibility of organisms to IFNs could provide important information to improve the therapeutic index of IFNs in terms of increased efficacy and reduced toxicity.

Ključne besede: *interferoni; kronobiologija; cirkadiana ritmičnost; protitumorska aktivnost; mielosupresivna aktivnost*

Izvleček – Izhodišča. *Kronobiologija preučuje biološke spremembe v določenem časovnem obdobju. Cikične spremembe različnih bioloških vrednosti, ki se ponavljajo vsakih 24 ur, imenujemo cirkadiane ritme. Cirkadiani ritmi, ki jih natančno uravnava hipotalamična biološka ura, so usklajeni z zaporednim menjavanjem dneva in noči. V cirkadianih ciklusih se spreminjata aktivnost in toksičnost citostatikov, antibiotikov in mnogih drugih učinkovin.*

Interferonski sistem je pomemben neimunski obrambni kompleks, ki ga sestavljajo tri različne vrste interferonov (IFNov): IFN-alfa, IFN-beta in IFN-gama. Vse tri vrste IFNov preprečujejo znotrajcelično razmnoževanje virusov, upočasnjujejo razmnoževanje tumorskih celic in krmilijo imunski odziv. Z IFNi zdravijo nekatere virusne in rakave bolezni ter imunske pomanjkljivosti. Klinično uporabnost IFNov omejujejo nezaželeni stranski učinki, med drugim tudi okvare kostnega mozga, jeter in živčevja. V poteku nekaterih kliničnih raziskav so opazili, da je intenziteta negativnih učinkov IFNov odvisna od časa dajanja IFNskih pripravkov.

Metode. *Pričujoči članek predstavlja kratek pregled kronobioloških raziskav na mišjem eksperimentalnem modelu. Poskusnim mišim smo ob različnih časih vbrizgavali IFNe in ugotavljali protitumorsko in mielotoksično dejavnost. Protitumorsko dejavnost smo ugotavljali z merjenjem časa preživetja miši s tumorji. Mielotoksično dejavnost smo ugotavljali s štetjem belih krvnih celic v periferni krvi miši in s štetjem kolonij matičnih celic granulocitne in makrofagne vrste v kostnem mozgu miši.*

Rezultati. *V prvem sklopu kronobioloških poskusov smo ugotovili, da je protitumorska dejavnost IFN-alfa in IFN-gama odvisna od časa vbrizgavanja. Poskusne miši z B-16 melanomskimi tumorji so preživele najdlje (55% daljše preživetje kot kontrolne miši), če smo jim vbrizgavali rekombinantni humani IFN-alfa (rHuIFN-alfa A/D) ob 4 HALO (Hours After Light Onset), to je 4 ure po vključitvi svetlobe. Če smo vbrizgavali rHuIFN-alfa A/D ob 12 HALO, je bilo preživetje poskusnih miši najkrajše (30% daljše preživetje kot kontrolne miši). Če smo dajali poskusnim mišim z B-16 melanomskimi tumorji rekombinantni murini IFN-gama (rMuIFN-gama), pa je bil čas preživetja najdaljši po vbrizgavanju ob 16 HALO (49% daljše preživetje kot kontrolne miši) in najkrajši po vbrizgavanju ob 8 HALO (23% daljše preživetje kot kontrolne miši).*

V drugem sklopu kronobioloških poskusov smo ugotavljali hematološko toksičnost različnih IFNskih vrst. Število belih krvnih celic v periferni krvi miši se je zmanjšalo najbolj po vbrizgavanju rHuIFN-alfa A/D ob 0 HALO (67% števila belih krvnih celic v

periferni krvi kontrolnih miši) in najmanj po vbrizgavanju ob 8 HALO (94% števila belih krvnih celic v periferni krvi kontrolnih miši). Vbrizgavanje rMuIFN-gama je povzročilo največji padec števila belih krvnih celic ob 4 HALO (70% števila belih krvnih celic v periferni krvi kontrolnih miši) in najmanjši padec ob 16 HALO (99% števila belih krvnih celic v periferni krvi kontrolnih miši). S testom za dokaz proliferacije matičnih celic granulocitne in makrofagne vrste smo dokazali, da je periferni hematotoksični učinek obeh IFNskih vrst posledica zaviranja razmnoževanja matičnih celic kostnega mozga in da je tudi stopnja supresije kostnega mozga (mielosupresija) odvisna od tega, kdaj v cirkadianem ciklusu vbrizgavamo rHuIFN-alfa A/D ali rMuIFN-gama.

Zaključki. *Zaključiti je mogoče, da omenjeni rezultati dokazujejo, da vbrizgavanje IFNov ob empirično izbranih časih v cirkadianem ciklusu povečuje protitumorski učinek in zmanjšuje hematotoksični učinek IFNov v poskusnih miših. Z natančnim poznavanjem ritmičnega spreminjanja dejavnosti interferonov bo mogoče bolje razumeti delovanje interferonskega sistema in natančno določiti čas, ko imajo IFNi največji terapevtski indeks.*

Introduction

Chronobiology explores the temporal relationship of biological phenomena (1). Virtually all physiological functions or parameters in living organisms exhibit a circadian rhythmicity i.e. they vary along the 24-hr scale. For example, body temperature or blood pressure; serum levels of different blood constituents, including proteins and electrolytes; the number and reactivity of circulating blood cells; and, mitotic indices of various kind of cells vary predictably as a function of time (1, 2).

The circadian rhythms are generated by an internal pacemaker, called the biological clock. In mammals the biological clock is located in the suprachiasmatic nuclei of the anterior hypothalamus (3). The timing of biological clock is modulated by environmental factors. In the case of circadian rhythmicity, the solar light – dark cycle is the most important external synchronizer.

Due to rhythms in physiological functions in man and experimental animals, it is expected that the positive and negative effects of most, if not all, known drugs will vary with the time of their administration. Indeed, a wide variety of drugs such as beta-blockers, antihistamines, antibiotics, and cytostatics exhibit daily variations in their effects (4).

Circadian rhythms in effectiveness and toxicity have been shown also for some biological response modifiers, e.g. interleukin-2, and tumor necrosis factor (5).

Interferons (IFN-alpha, IFN-beta, and IFN-gamma) are a group of cellular proteins that have direct or indirect antiviral and antitumor effects. As a part of the complex cytokine network, they also exert a multitude of immunoregulatory activities. Thus, it is not surprising that IFNs have been established as useful therapeutic agents in clinical medicine. They are used for the treatment of different viral and malignant diseases, and immune disorders (6). Unfortunately, the clinical effectiveness of IFNs is lower than expected on the basis of in vitro observations. In addition, administration of IFNs frequently induces toxic side effects which include an influenza-like syndrome, fatigue, somnolence, anorexia, hypotension, thrombocytopenia, and leukopenia.

In order to increase the therapeutic index (i.e. to minimize the side effects and maximize the effectiveness) of IFNs, different approaches have been suggested and investigated. The chronobiological approach stresses the importance of the timing of administration of IFNs. So far only a few preclinical and clinical studies with IFNs have been conducted.

Mann et al. (7) demonstrated in a murine model that administration of either IFN-alpha/beta or IFN-gamma in the evening (2100 hr) resulted in a longer and more extensive reduction of the number of peripheral blood leucocytes than when IFNs were administered in the morning (0900 hr).

Levi et al. (8) examined the ability of IFN alpha/beta to stimulate mouse splenocyte NK cell activity at different circadian stages. NK cells harvested in the second half of the active (dark) span or in the early rest (light) span of mice exhibited maximal activation by IFN.

Indiveri and Puppo (9) injected healthy individuals with IFN-alpha in the morning (0800 hr) and in the evening (2000 hr) and showed that only the morning injections altered the physiological circadian rhythm of the circulating count of T cells and NK cells.

Abrams et al. (10) reported their clinical observation that most of their patients experienced less fatigue and fewer acute toxic effects when recombinant IFN-alpha was administered in the evenings rather than in the mornings.

In another clinical study, Brummer-Depres et al. (11) described a reduction in negative side effects of recombinant human IFN-alpha therapy in patients with metastatic carcinoma when their continuously infusing interferon pump was programmed to deliver the maximum amount of IFN during the time interval of 1800 hr to 2200 hr.

In order to study in more details the putative time dependence of the effectiveness and/or toxicity of IFNs, we employed a murine experimental model and evaluated the antitumor activity and the myelotoxicity of IFN-alpha and IFN-gamma administered at six different times in the circadian cycle (12–14).

1. CHRONOBIOLOGICAL EFFECT ON IFN-MEDIATED ANTITUMOR ACTIVITY

The length of the survival of tumor-bearing mice was used as an indicator of IFN antitumor activity and evaluated for different times of IFN administration (12).

Materials and methods

Female C57Bl/6 mice were housed in two different isolated animal rooms with alternating cycles of 12 hrs of light (L) and 12 hrs of darkness (D). Food and water was freely available. After two to three weeks of adaptation, mice were randomly divided into groups of 8 to 10 animals each. They were intraperitoneally injected with 10^6 B16 melanoma cells at six different times in the circadian cycle (0, 4, 8, 12, 16, or 20 hours after light onset [HALO]). Exactly 24 hours later, at the same circadian stages, the mice

received intraperitoneal injections of recombinant human IFN-alpha A/D (rHuIFN-alpha A/D) (10,000 U/day) and recombinant murine IFN-gamma (rMuIFN-gamma) (2,000 U/day). Control mice were injected with bovine serum albumin in phosphate buffered saline (BSA/PBS). Treatments were given for five days. The mice were monitored for day of death and the average day of death was determined. The percent increased life span (ILS) was calculated by the following formula:

%ILS = [(day of death for IFN-treated mice – average day of death for control mice) : (average day of death for control mice)] × 100

Results

Figure 1 presents the averaged results of three identical experiments. Percent increased life span (ILS) is plotted against the time of IFN administration. It is evident that the antitumor activity of rHuIFN-alpha A/D (Panel A) and rMuIFN-gamma (Panel B) varied with the time of IFN treatment. Maximum %ILS (55%) occured when rHuIFN-alpha A/D treatment was given at 4 HALO. Minimum % ILS (30%) occured when rHuIFN-alpha A/D was given at 12 HALO. Mathematical evaluation of the antitumor activity of different concentrations of rHuIFN-alpha A/D administered at 4 HALO and 12 HALO showed that rHuIFN-alpha A/D was about five times more potent when administered at 4 HALO. After the administration of rMuIFN-gamma, maximum %ILS (49%) occured at 16 HALO and minimum %ILS (23%) at 8 HALO. Mathematical evaluation of the antitumor activity of different concentrations of rMuIFN-gamma showed that rMuIFN-gamma was about nine times more potent when it was administered at 16 HALO.

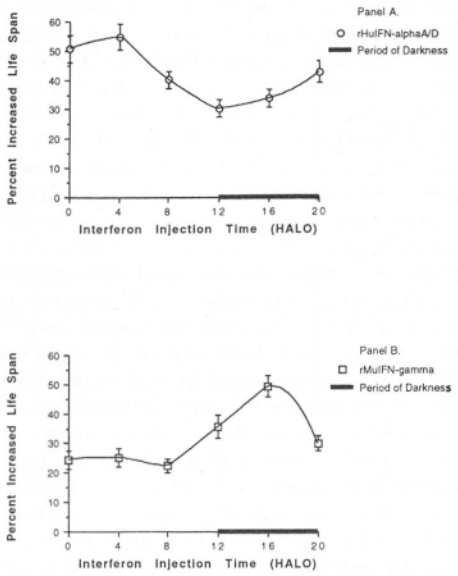

Fig. 1. *Circadian rhythmicity in the antitumor effect of rHuIFN-alpha A/D and rMuIFN-gamma. Mice kept in a LD 12 : 12 cycle were inoculated intraperitoneally with 10^5 B16 melanoma cells at 6 different time points in the circadian cycle. Beginning exactly 24 hrs after tumor cell inoculation, mice were given intraperitoneal injections of either IFN or BSA/PBS. Treatments were given for 5 days. The mice were monitored for day of death. The results are plotted as percent increased life span versus the time of treatment. Panel A: Results observed for treatment with rHuIFN-alpha A/D (10,000 U/day). Panel B: Results observed for treatment with rMuIFN-gamma (2,000 U/day). Each data point represents the mean ± SE of the average day of death from three experiments.*

2. CHRONOBIOLOGICAL EFFECT ON IFN-MEDIATED MYELOSUPPRESIVE ACTIVITY

Peripheral white blood cell (WBC) suppression and bone marrow suppression were used as indicators of IFN myelotoxic activity and evaluated for different times of IFN administration (13, 14).

Materials and methods

Female C57Bl/6 mice were kept under the same conditions and lighting regimen as described above. After two to three weeks of adaptation, mice were randomly divided into groups of 8 to 10 animals each. They were injected intraperitoneally with 4,000 U/day of rHuIFN-alpha A/D or with 500 U/day of rMuIFN-gamma for four consecutive days, each day at six time points spanning 24 hr (0, 4, 8, 12, 16, or 20 HALO). Control mice were injected with BSA/PBS. The mice were bled on day 5, and the total WBC count was determined for each mouse. Bone marrow function was measured in a granulocyte-macrophage colony-forming unit (GM-CFU) assay (15). The mice were sacrificed and bone marrow cells were extracted from the femurs. Bone marrow cells were resuspended in a semisolid culture medium (alpha MEM containing 15% bovine serum, antibiotics, and 0.35% agarose) and plated onto 35-mm plastic gridded dishes containing recombinant murine granulocyte-macrophage colony-stimulating factor (rMuGM-CSF). After seven days of incubation (37°C; 7% CO_2) colonies containing 50 or more cells were enumerated.

Results

Figure 2 presents the averaged results of two identical experiments. Peripheral WBC count is plotted against the time of IFN administration. It is evident that injection of control mice with BSA/PBS at various HALO had no significant differential effect on the peripheral WBC counts. Furthermore, the data show that the

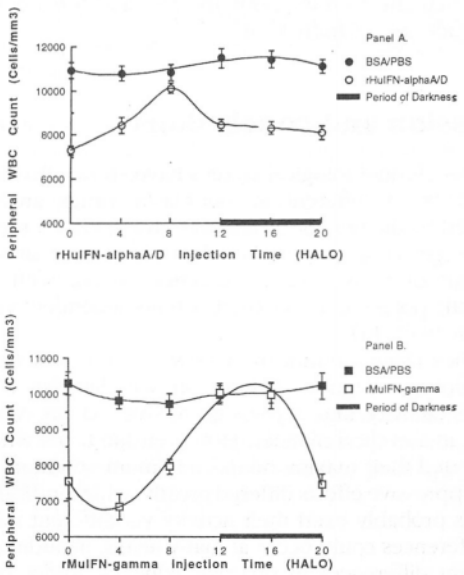

Fig. 2. *Chronobiological effect of rHuIFN-alpha A/D and rMuIFN-gamma on peripheral WBC counts. Mice kept in a LD 12 : 12 cycle were injected subcutaneously with either IFN or BSA/PBS at 6 different time points in the circadian cycle. The mice were bled on day 5 after 4 days of treatment. The number of peripheral WBCs was counted in a hemacytometer. The results are plotted as peripheral WBC count versus the time of treatment. Panel A: Results observed for treatment with rHuIFN-alpha A/D (4,000 U/day). Panel B: Results observed for treatment with rMuIFN-gamma (500 U/day). Each data point represents the mean ± SE of WBC counts from two experiments.*

peripheral WBC suppressive effect of rHuIFN-alpha A/D (Panel A) and rMuIFN-gamma (Panel B) varied with the time of the IFN treatment. The treatment with rHuIFN-alpha A/D at 8 HALO had very little peripheral WBC-suppressive effect, with WBC counts at 94% of the value observed for control mice. In contrast, the treatment at 0 HALO had the greatest peripheral WBC-suppressive effect, with WBC counts at 67% of the value observed for control mice. Treatment with rMuIFN-gamma at 12 HALO and 16 HALO had no or very little peripheral WBC-suppressive effect, with WBC counts at 101% and 99% of the value observed for control mice respectively. In contrast the treatment with rMuIFN-gamma at 4 HALO had the greatest peripheral WBC-suppressive effect, with WBC counts at 70% of the value for control mice.

Using the GM-CFU assay, bone marrow function was also shown to be differentially suppressed by treatment with rHuIFN-alpha A/D and rMuIFN-gamma in a manner parallel to that seen with peripheral WBCs (data not shown). Bone marrow cells from mice treated with rHuIFN-alpha A/D at 0 HALO (the time of maximum reduction of the peripheral WBC count) showed an average of 39% suppression in the number of granulocyte/macrophage colonies developing for each of the various concentrations of rMuGM-CSF employed. In contrast, the responsiveness of bone marrow cells from mice treated with rHuIFN-alpha A/D at 8 HALO (the time of minimum reduction of the peripheral WBC count) was not significantly different from that of the control mice treated with BSA/PBS (an average of 5% suppression). Bone marrow cells from mice treated with rMuIFN-gamma at 4 HALO (the time of maximum reduction of the peripheral WBC count) showed an average of 48% suppression in the number of granulocyte/macrophage colonies developing in the presence of rMuGM-CSF. In contrast, the responsiveness of bone marrow cells from mice treated with rMuIFN-gamma at 14 HALO (the time of minimum reduction of the peripheral WBC count) was similar to or higher than that of control mice (an average of -8% suppression). Thus, the variation in peripheral WBC counts seen at different HALO was reflective of a differential effect on the bone marrow and was not just due to an alteration in cell trafficking.

Discussion and conclusions

Numerous chronobiological studies have shown that the efficacy and toxicity of different antineoplastic drugs are selectively influenced by the time of their administration (for a recent review see 16). A growing amount of evidence indicates that IFNs, which are a part of body's natural defence system with substantial therapeutic potential, also exhibit a time-dependent variations in their activity (7–14).

Our studies, using a murine model system, and employing two IFN types (alpha and gamma) have shown that the IFN-induced antitumor activity and myelosuppressive activity vary in their intensity in a cyclical manner. However, the times when the two IFNs exerted their maximum and minimum antitumor as well as myelosuppressive effects differed greatly, which indicates that the two IFNs probably exert their activity via different mechanisms. Such differences could occur at many levels, including circadian-dependent differences in pharmacokinetics, tumor blood flow, tumor responsiveness, and host immune responsiveness. It is also possible that the two IFNs block tumor cell and bone marrow cell division at different stages of the cell cycle.

Table 1 shows the injection times associated with maximum and minimum antitumor activity as well as maximum and minimum myelosuppressive activity of both IFNs. It is evident that the time of minimum myelosuppressive activity of rHuIFN-alpha A/D was at 8 HALO, while the time of maximum antitumor activity was at 4 HALO. The time of minimum myelosuppressive activity of rMuIFN-gamma was at 14 HALO, while the time of maximum antitumor activity was at 16 HALO. These results indicate that administration of rHuIFN-alpha A/D at about 6 HALO and of rMuIFN-gamma at about 15 HALO would provide the highest therapeutic index for the antitumor activity of both IFNs in mice. It is important to confirm these studies for other tumors in the mouse system.

Tab. 1. *Comparison of circadian-dependent antitumor and myelosuppressive activities of IFNs.*

Treatment	Hours after light onset			
	Myelosuppressive activity		Antitumor activity	
	Maximum	Minimum	Maximum	Minimum
rHuIFN-alpha A/D	0	8	0–4	12–16
rMuIFN-gamma	4	14	16	0–8

It is difficult to extrapolate the results from the murine to the human system. However, if we assume the diurnal habits of humans (16 h awake : 8 h sleep schedule with the sleep period from 11 PM to 7 AM) versus the nocturnal habits of mice (12 h awake : 12 h sleep schedule with the sleep period from 0 HALO to 12 HALO), the best time to administer rHuIFN-alpha would be at about 3 AM and the best time to administer rMuIFN-gamma would be at about 11 AM.

The results of these studies support the concept that administration of IFNs at specifically determined time points in the circadian cycle may reduce the side effects and increase the efficacy of IFNs in man.

References

1. Pauly JE. Chronobiology: anatomy in time. Am J Anat 1983; 168: 365–88.
2. Haus E, Lakatua DJ, Swoyer J, Sackett-Lundeen L. Chronobiology in hematology and immunology. Am J Anat 1983; 168: 467–517.
3. Stephan FK, Zucker I. Circadian rhythms in drinking behaviour and locomotor activity of rats are eliminated by hypothalamic lesions. Proc Nat Acad Sci USA 1972; 69: 1583–6.
4. Lemmer B, Labrecque G. Chronopharmacology and Chronotherapeutics: Definitions and concepts. Chronobiol Intern 1987; 4: 319–29.
5. Levi F, Canon C, Depres-Brummer P et al. The rhythmic organization of the immune network: implications for the chronopharmacologic delivery of interferons, interleukins and cyclosporin. Adv Drug Deliv Rev 1992; 9: 85–112.
6. Baron S, Tyring SK, Fleischmann WR Jr. et al. The interferons: mechanisms of action and clinical applications. JAMA 1991; 266: 1375–83.
7. Mann EA, Markovic SN, Murasko DM. Inhibition of lymphocyte recirculation by murine interferon: effects of various interferon preparations and timing of administration. J Interferon Res 1989; 9: 35–51.
8. Levi F, Canon C, Di Palma M, Florentin I, Misset J-L. When should the immune clock be reset? From circadian pharmacodynamics to temporally optimized drug delivery. Ann NY Acad Sci 1991; 618: 312–29.
9. Indiveri F, Puppo F. Neuroendocrine effects of biologic response modifiers. In: Proceedings of the 16th International Congress of Chemotherapy, Jerusalem, 1989; 324.
10. Abrams PG, McClamrock E, Foon KA. Evening administration of alpha interferon. N Engl J Med 1985; 312: 443–4.
11. Brummer-Depres P, Levi F, Di Palma M et al. A phase I trial of 21-day continuous venous infusion of alpha-interferon at circadian rhythm modulated rate in cancer patients. J Immunother 1991; 10: 440–7.
12. Koren S, Whorton EB Jr., Fleischmann WR Jr. Circadian dependence of interferon antitumor activity in mice. J Natl Cancer Inst 1993; 85: 1927–32.
13. Koren S, Fleischmann WR Jr. Circadian variations in myelosuppressive activity of interferon-alpha in mice: identification of an optimal treatment time associated with reduced myelosuppressive activity. Exp Hematol 1993; 21: 552–9.
14. Koren S, Fleischmann WR Jr. Optimal circadian timing reduces the myelosuppressive activity of recombinant murine interferon-gamma administered to mice. J Interferon Res 1993; 13: 187–95.
15. Bradley TR, Metcalf D. The growth of mouse bone marrow cells in vitro. Aust J Exp Biol Med Sci 1966; 44: 287–99.
16. Hrushesky WJM, Martynowicz M, Markiewicz M, Von Roemeling R, Wood PA, De la Pena SS. Chronotherapy in cancer: a major drug-delivery challenge. Adv Drug Deliv Rev 1992; 9: 1–83.

Professional article/Strokovni prispevek

PORCINE INTERFERON GAMMA (PoIFN Gamma)

PRAŠIČJI INTERFERON GAMA (PoIFN Gama)

Bratko Filipič[1], Sonja Rozman[2], Katarina Carlsson[3], Avrelija Cencič[4]

[1] Institute of Microbiology, Medical Faculty, Zaloška 4, 61105 Ljubljana, Slovenia
[2] BIA d.o.o., Teslova 30, 61000 Ljubljana, Slovenija
[3] Centre of Chemistry, University of Lund, 22100 Lund, Sweden
[4] High School of Agronomy, University of Maribor, Vrbanska 30, 62000 Maribor, Slovenija.

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Key words: *porcine interferon; human interferon; preparation; purification; characterization*

Abstract – Background. *The experiments were performed to study the induction, partial purification and antigenic analysis of PoIFN Gamma, as well as to prepare the potentially clinically useful form for in vivo experiments.*

Methods. *Porcine buffy-coat was resuspended in the Eagle's medium in concentration of 10^7–10^8 cells/ml. The induction was performed by the LCL (Lens culinaris lectin) in concentration of 25 µg/ml. PoIFN Gamma was partially purified by the adsorption/elution on the water glass, and than further on with the column chromatography on the CPG (control pore glass). The IFN obtained was characterized through the following tests: antiviral/antiproliferative activity, stability at pH 2.0 and 56°C, protein content, serological tests and Agarose gel electrophoresis.*

Results. *Using the LCL as inducer, about 36,000 units of the antiproliferative activity/ml of PoIFN Gamma were obtained. Through the adsorbtion/elution technique on the water glass, the purification degree between 7 and 10 times was found. This was enlarged 100 times after an chromatography/rechromatography on the CPG (control pore glass). The agarose gel electrophoretic profile shows that the most of the interferon activity can be found in the Alpha-2 like fraction. The results of the serological analysis shows that the cross reactivity between the PoIFN Gamma and HuIFN Alpha but not with HuIFN Gamma can be found.*

Conclusions. *The studied PoIFN Gamma shows the cross reactivity with HuIFN Alpha as well as with its natural subtype Alpha 2. Further experiments with purified PoIFN Gamma together with C-terminal sequence comparison are expected to show the real level of similarity between the Porcine and Human IFNs.*

Ključne besede: *prašičji interferon; humani interferon; preparacija; purifikacija; karakterizacija*

Izvleček – Izhodišča. *Preučevanje indukcije, delnega čiščenja in antigenske analize prašičjega imunskega interferona ter njegova primerjava s humanimi interferoni in njihovimi naravnimi podtipi.*

Metode. *Prašičjo levkomaso smo resuspendirali v Eaglovem gojišču v koncentraciji 10^7–10^8 celic/ml in inducirali IPN z dodatkom 25 µg/ml lektina LCL (Lens culinaris lectin). Delno čiščenje smo vršili s pomočjo adsorpcije/elucije na vodnem steklu, in nato v nadaljevanju s kolonsko kromatografijo na poroznem steklu. Dobljen interferonski preparat smo ovrednotili s pomočjo naslednjih testov: meritve protivirusne in proticelične aktivnosti, stabilnosti pri pH 2,0 in 56°C, vsebnosti beljakovin, seroloških testov in elektroforetskega profila v agaroznem gelu.*

Rezultati. *Največje količine imunskega interferona dobimo s pomočjo LCL kot induktorja, pri čemer dobimo približno 36.000 enot proticelične aktivnosti/ml. Z uporabo metode adsorpcije/elucije na vodnem steklu interferon očistimo 7–10-krat. Z nadaljnjo kromatografijo in rekromatografijo na poroznem steklu pa še 100- krat. Elektroforetski profil kaže, da večino interferona dobimo v Alfa-2 podobni frakciji. Serološke analize pokažejo sorodnost s humanim levkocitnim interferonom, ne pa s humanim imunskim.*

Zaključki. *Dobljen interferonski preparat kaže sorodnost s humanim levkocitnim interferonom oz. z njegovim naravnim podtipom Alfa 2. Z izpopolnjeno metodo čiščenja in sekvencioniranja prašičjega imunskega interferona bo možna primerjava s humanim interferonom.*

Introduction

Similar interferon systems as in human can be found in other animal species as: mouse, rat, horse, bovine, pig, monkey etc. Among others, porcine interferons (PoIFNs) became the subject of interest (1, 2) because of the relatively high antigenic similarity between porcine and human. The similarity between the Hu and PoIFNs is about 78.5% (at nucleotide level) (3) in case of Alpha. When Gamma IFNs were compared (rPoIFN Gamma/rHuIFN Gamma), the homology was estimated at 58% (4).

Only recently when from both PoIFNs (Alpha and Gamma) the recombinant forms became available, the low clinical usefulness in veterinary clinics was observed, even if the high doses were administered to the animals. When the natural (nonrecombinant) forms of IFNs with approximately ten times lower doses were used, better clinical effects were achieved. Similar results were

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obtained when HuIFNs were used in clinics. Further more, in cases when million-unit dosages of rIFNs were used on long-term treatment of cancer patients neutralising antibodies arose in patients' sera.

With new experimental data it becomes more and more evident that for good clinical results in animals and also in humans the natural form of IFNs should be used (5, 6).

Antiviral and antiproliferative/antitumor activity can be enhanced by Alpha/Gamma combinations in the porcine system (7, 8). Especially in the case of the antiproliferative activity, the synergism between HuIFNs Alpha and PoIFN Gamma was observed, when these were mixed in the ratio of 500 AV units of HuAlpha and 100 AV units of PoGamma. Such data point out the possible clinical exploitation of natural PoIFNs forms (Alpha/Gamma) in human and veterinary medicine, even though the importance of possible antigenic differences should be taken into account.

The experiments presented were performed to study the induction, partial purification and antigenic analysis of the PoIFN Gamma, as well as to prepare the potentially clinically useful form for the in vivo experiments in the pigs.

Materials and methods

Blood collection and buffy-coat preparation

Porcine blood was collected aseptically into sterile flasks containing citrate (3.8% of Sodium citrate) to prevent coagulation. After sedimenting the erythrocites, the buffy-coat was separated by introducing the 30% sacharose. The upper part containing the buffy-coat and plasma was centrifuged for 20 minutes at 2500 RPM (Rotation per minutes). The sedimented buffy-coat was washed twice with saline and finally resuspended in the Eagle's medium containing 4% of porcine plasma and antibiotics.

Cultivation and interferon induction

Porcine buffy-coat was resuspended in the Eagle's medium containing 4% of the homologous plasma and antibiotics in a concentration of 10^7–10^8 cells/ml. Induction was performed by addition of LCL (Lens culinaris lectin) in a concentration of 25 µg/ml (9,10). After three days of cultivation on spinner flask of the volume of 2000 ml (37°C, 120–140 RPM) cells were sedimented by centrifugation at 2500 RPM for 20 minutes. The supernatant obtained was used for further purification and analyses. The remaining sediment (cells) was discarded.

Partial purification of PoIFN Gamma

Interferon was isolated from the supernatant as follows: to 100 ml of IFN-containing supernatant, 2g of autoclaved SiO_2 (water glass) were added and incubated overnight at +4°C. On the next day, the suspension was centrifuged at 2500 RPM for 30 minutes to sediment the water glass. The sedimented water glass was resuspended in the 1/8 of original volume of the mixture of 50% monoethylene-glycole in 1.4 mM NaCl to elute interferon. After 2 hours the water glass was sedimented by centrifugation (2500 RPM/30 minutes) and the same procedure in 1/16 of the original volume of the mixture described above was repeated. Both eluates were mixed with 0.1% of FCS (foetal calf serum) and dyalised against distilled water.

CPG chromatography

Interferon obtained after the batch purification was further purified by CPG (control-pore-glass) (Sigma) as follows: The columns (5cm in diameter/30cm in lenght) were filled with the CPG in Tris-NaCl, pH 6.8. After the overnight equilibration, the IFN samples were put into the column and the elution was performed with the same buffer (pH = 8.6). The fractions (5ml) were collected and tested for: protein content, antiviral and antiproliferative activity.

Measurement of antiviral/antiproliferative activity

Each of the samples of IFN was tested for antiviral (AV) activity by 50% cytopathogenic inhibition assay on FL cells with HSV1 (Herpes simplex virus type 1) as challenge virus (11). HuIFN Alpha (Institute for Immunology, Zagreb, Croatia and EGIS, Budapest, Hungary) at 1,000 AV units/ml was used as a standard. Cell growth inhibition assay was performed on FL and HEF cells (12). GI/C (Growth index/Control) in % was calculated from a line generated by dose response curve for HuAlpha and PoGamma.

Stability tests

To determine the nature of IFNs, samples were exposed to pH 2.0 and heating at 56°C for 20 minutes. The antiviral/antiproliferative activity was determined before and after the treatment.

Protein content

In each sample, the quantity of the proteins was determined by a modified Lowry method (14).

Agarose gel electrophoresis

To test the purity of interferons, the agarose gel electrophoresis in Veronal buffer, pH 8.6/200 V for 1 hour, was performed. After completing the electrophoresis the gels were stained with Coomassie brilliant blue R-250 and destained in the mixture of methanol:acetic acid:water (1:2:1). The gel was then dried and photographed.

Serological analyses

To determine serological similarities/differences between PoIFN Gamma and HuIFNs, the "constant method" (13) was used as follows: to the FL cells in the microtiter plates, first a constant dillution of antiserum was added, and then two-fold dillutions of IFNs with the virus (HSV1). In parallel, a simple IFN titration was performed. The neutralisation index (NI) was calculated as follows:

$$NI = \log_3 (\text{Antiserum} + \text{Interferon}) - \log_3(\text{Interferon})$$

Results

Interferon induction

According to our previous data (9), the best yield of IFN could be obtained when LCL (Lens culinaris lectin) in a concentration of 25 ug/ml was used as inducer. The average titer of 36.000 AP units/ml could be obtained. After comparing the AV/AP ratio between HEF and FL cells (Tab. 1), it was established that in porcine system 1 AV (antiviral unit) corresponds to 10 AP (antiproliferative units). In human system, 1 AV unit corresponds to approximately 20 AP

Tab.1. *AP (antiproliferative) units of 1,000 AV (antiviral) units of porcine mitogen induced interferon (PoIFN Gamma) and HuIFN Alpha.*

	AP units on	
1,000 AV units of	HEF[1]	FL[2]
PoIFN Gamma	10,500	10,000
HuIFN Alpha	18,000	20,000

[1] HEF = Human embryonal fibroblasts (Nontransformed)
[2] FL = Human amniotic cell line (Transformed)

units. Based on the above data, the antiproliferative (AP) units were defined as the quantity of IFN causing the 50% inhibition of the GI/C either on FL or HEF cells.

Partial purification of PoIFN Gamma

The data obtained (Tab. 2) show that the batch purification method is simple and effective. First of all, it is a single step with the degree of purification between 7 and 10 times. Through the batch purification the bulk of protein impurities were removed, so that after the additional step of CPG chromatography almost pure IFN preparations could be obtained without serious loss of biological activity (antiviral/antiproliferative).

Tab. 2. *SiO_2 (Water glass) purification of PoIFN Gamma.*

Step	Volume (ml)	IFN AP/ml[1]	IFN Tot[2]	Protein mg/ml	Protein Tot.	Spec. act.[3]	Degree of purif.[4]
Crude[5]	1000	8.44[6]	8.44	5.63	5360	1500	0
Final I[7]	125	9.99	1.25	0.73	91.0	11639.3	7.76
Final II[8]	125	13.40	1.66	0.73	91.0	15466.3	10.30

[1] AP/ml = Antiproliferative units/ml
[2] Total AP units x 10^6
[3] Specific activity = AP units/mg of proteins
[4] Degree of purification for final I = Specific activity of final I / Specific activity of crude
Degree of purification for final II = Specific activity of final II / Specific activity of crude
[5] IFN containing supernatant
[6] IFN containing supernatant
[7] Final I = first eluate from SiO_2
[8] Final II = Second eluate from SiO_2

Column chromatography

CPG chromatography was performed in two steps: Chromatography and rechromatography (Fig. 1, Fig. 2). Chromatographic profile shows, that the most of the active IFNs molecule can be found between fractions 30 and 60. The bulk of impurities (other proteins) were in fractions 15–29. When the active fractions (30–60) from the chromatography were collected and concentrated on Amicon YM-10 and rechromatographed on the same column, nearly all impurities were removed. The average reduction of non-IFN proteins from 2.8 mg/ml in chromatography to 0.06 mg/ml in rechromatography was found.

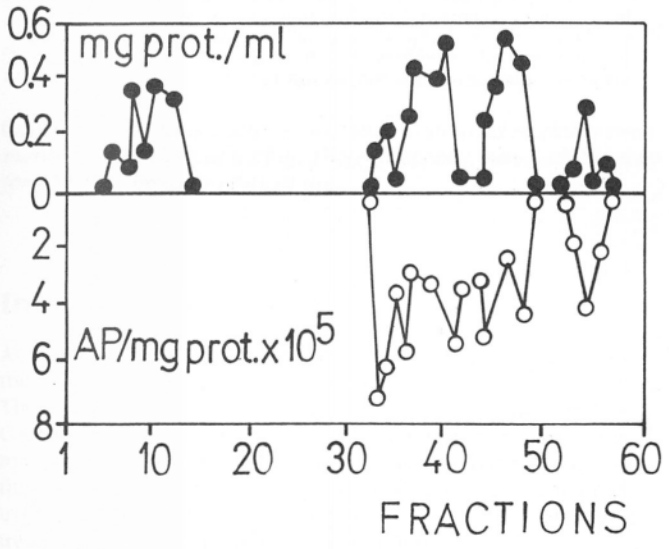

Fig. 1. *CPG (control pore glass) chromatography of porcine interferon gamma (PoIFN Gamma).*

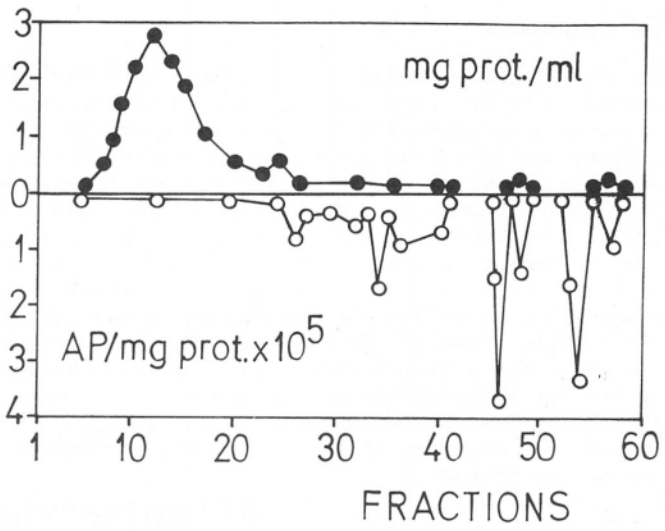

Fig. 2. *Rechromatography of porcine interferon gamma (PoIFN Gamma) on CPG (control pore glass).*

Agarose electrophoresis

IFN samples were put onto the electrophoresis in the Agarose gel (Fig. 3). Comparison of the data obtained shows that most of the IFN's activity (AV/AP) can be found in the Alpha 2-like fraction (in comparison with the immunoglobuline electrophoretic profile on the Agarose electrophoresis).

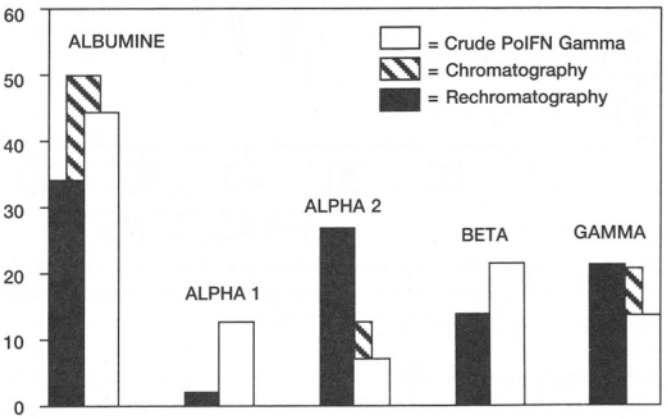

Fig. 3. *Agarose gel electrophoresis of porcine interferon gamma (PoIFN Gamma) at different level of CPG (control pore glass) purification.*

Serological analysis

The results of the serological analyses (Tab. 3) show the similarity (cross reactivity) between PoIFN Gamma and HuIFN Alpha (NI= – 1.15), as well as with HuIFN Alpha 2 (NI= – 0.29).

Tab. 3. *Some characteristics of PoIFN Gamma (porcine mitogen induced interferon) in comparison to PoIFN Alpha and HuIFNs Alpha and Gamma.*

IFN type:	pH 2.0	56°	Reaction with Anti-[1] Po-Gamma NI	Hu-Gamma NI	Hu-Alpha NI	Hu-Alpha 1 NI	Hu-Alpha 2 NI
PoIFN-Gamma	Stable	Stable	–1.15	0	–1.94	0	–0.29
PoIFN-Alpha	Labile	Labile	0	0	–2.43	0	–0.53
HuIFN-Gamma	Labile	Labile	0	–0.98	0	0	0
HuIFN-Alpha	Stable	Stable	–0.96	0	–2.85	–2.00	–1.60

[1] NI = (Neutralisation index) = $\log_3$ (IFN titre + antiserum) – $\log_3$ (IFN titre)

Discussion

The data obtained throughout the experiments show that the method developed herein gives the final product (PoIFN Gamma), which can be further tested by the in vivo experiments with pigs. Comparison of the PoIFN Gamma with other porcine IFNs (Alpha, SP1) show the difference in the antigenic properties. There is no cross-reactivity between them. When thermal stability and pH 2.0 resistance of PoIFNs oncompared, Alpha and SP1 are labile, whereas Gamma is stable like HuIFN Alpha. The antigenic similarity between Po and HuIFNs was studied on mice (13). Pirogenicity tests on rabbits (15) showed the picture similar to the HuIFN Alpha. Chromatographic data on CPG show the co-migration of the fractions giving the antiviral and antiproliferative activity (Fig. 4). A molecular heterogenicity of PoIFN Gamma, similar to that described for HuIFN Gamma (16), was suggested in Western blots of purified nPoIFN Gamma (17), even they are antigenically different.

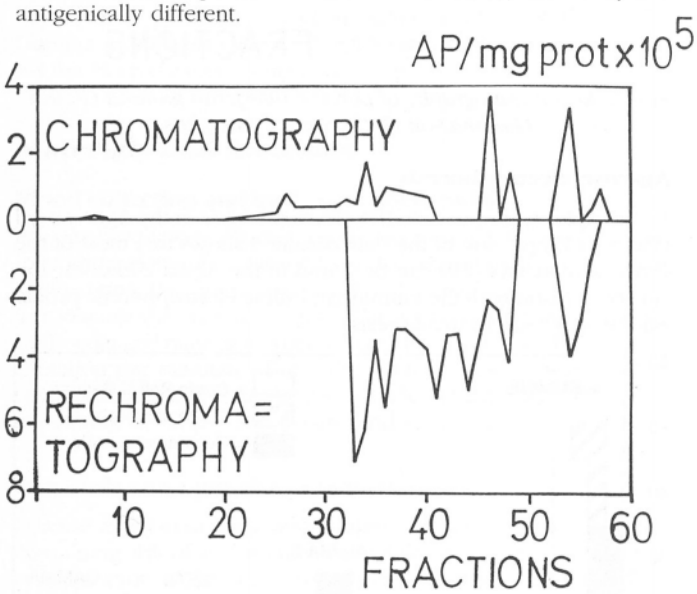

Fig. 4. *Comparison between chromatographic profile of crude PoIFN Gamma profile and rechromatography of biologically active fractions on the CPG column.*

As for the induction with LCL, the yield is much higher after induction with the combination of PMA-PHA (16). The developed purification method can be simply enlarged. When CPG chromatography/re-chromatography is compared to ConA/Sepharose, the IFN's binding is stronger on CPG than on ConA, which suggests better output.

Simultaneously, we found (data not shown) that PoIFN Gamma can be successfully used in humans for treating herpetic infections (Herpes labialis) without any side effects. This suggests that the antigenic similarity between the pig and human is high enough, that PoIFN Gamma can be used in humans.

In future experiments with chromatofocusing and HPLC chromatography, the purity level that is necessary for amino acid sequencing and molecular characterisation of PoIFN Gamma should be achieved, and with all these data the final comparison of the similarity between PoIFN Gamma and HuIFN Alpha (Alpha 2) should be possible.

References

1. Richmond JY. An interferon like inhibitor of fouth-and-mouth disease virus induced by phytohaemmaglutinine in swine leukocyte cultures. Arch Ges Virusforsch 1969; 27: 282–9.
2. Soloviev VD, Ogarkov VI, Marchenko VI, Monastyreva LN et al. Swine blood leukocytes, a new source of production of interferon active in human cells. Vopr Virusol 1980; 25: 716–20 (in Russian).
3. Lefevre F, LaBonnardiere C. Molecular cloning and sequence of a gene encoding biologically active porcine Alpha interferon. J IFN 1986; 6: 349–60.
4. Charley B, McCullough K, Martinod S. Antiviral and antigenic properties of recombinant porcine interferon gamma. Vet Immunol Immunopathol 1987; 7: 357–68.
5. Lavrukhina LA, Gutman NR, Manakhanova IS. Antiviral effect of Swine leukocyte interferon in experimental mice. Vopr Virusol 1981; 20: 414–8 (in Russian).
6. Jereb B, Us-Kraševec M, Červek J, Šooš E. Intrapleural application of human leukocyte interferon (MLI) in breast cancer patients with ipsilateral pleural carcinomatosis. J IFN Res 1987; 7: 357–63.
7. Piasecki E. Properties of natural porcine interferons. J IFN Res 1988; 6: 349–60.
8. Filipič B, Golob A, Toth S, Mecs I, Beladi I, Likar M. Interactions between human and porcine interferons. Acta Virol 1991; 35: 19–26.
9. Filipič B, Carlsson K, Zupan S, Knežević M, Raspor P. Preparation and partial purification of porcine interferon Gamma (PoIFN Gamma). Acta Pharm 1993; 43: 71–4.
10. Filipčič B, Golob A, Struna T, Likar M. Porcine mitogen induced interferon. Acta Virol 1994; 38: accepted for publication.
11. Forti LR, Schuffman SS, Davies AH, Mitchell MW. Objective antiviral assay of the interferons by computer assisted data collection and analysis. In: Pestka S ed. Method in enzimology, Vol.119, part C, New York, London: Academic Press, 1986: 533–40.
12. Filipič B, Golob A, Sinadinovska R, Babič D, Struna A, Pretnar G. Preparing and purification of porcine immune interferon. In: Likar M ed. Posvetovanje ob 45. obletnici Inštituta za mikrobiologijo Medicinske fakultete v Ljubljani, Ljubljana: Inštitut za mikrobiologijo; 1990: 71–80.
13. LaBonnardiere C, Laude H, Berg K. Biological and antigenic relationship between virus-induced porcine and human interferons. Ann Inst Pasteur/Virol 1986; 137E: 171–80.
14. Filipič B, Carlsson K, Hartman-Pretnar K, Podgornik A, Koselj P. A novel protein determination micromethod. Acta Pharm 1992; 42: 355–60.
15. Soloviev VD, Pokidysheva LN, Volodnina TN. On antigenic similarity of some Alpha-interferons. Vopr Virusol 1982; 27: 526–8 (in Russian).
16. Yip YK, Roy HL, Urban C, Vilček J. Partial purification and characterization of human immune interferon. J Proc Natl Acad Sci USA 1981; 78: 1601–5.
17. Gonzales-Juarrero M, Mebus C, Garmendia EA. Porcine lymphocyte gamma interferon responses to mitogenic stimuli monitored by a direct immunoassay. Vet Immunol Immunopathol 1994; 40: 201–12.

Review article/Pregledni članek

TUMOR NECROSIS FACTOR – BIOLOGICAL CHARACTERISTICS AND APPLICATIONS IN CANCER THERAPY

TUMORSKI NEKROZNI FAKTOR – BIOLOŠKE ZNAČILNOSTI IN PROTITUMORSKO DELOVANJE

Gregor Serša

Institute of Oncology, Zaloška 2, 61105 Ljubljana

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Key words: *tumor necrosis factor; radiation therapy; chemotherapy*

Abstract – Background. *Cytokines modulate and/or mediate many essential biological processes, particularly those involved in cell growth, activation and differentiation. Although tumor necrosis factor (TNF) was originally discovered as a molecule with antitumor activity, it has recently been recognized for its pleiotropic function in the cytokine network. TNF is an important mediator of immune response and inflammation. It is also selectively cytotoxic to some malignant cells in vitro, but this property does not always correlate with its antitumor activity in vivo. Many tumors are sensitive in vivo to TNF treatment, although their tumor cells are in vitro TNF resistant. This indicates that TNF antitumor effects in vivo are host-mediated, via effects on tumor vasculature and stimulation of specific host immune antitumor response. Although TNF was demonstrated to be effective on solid tumors in animal models, results of clinical trials were disappointing. Very few objective tumor responses were recorded, with severe adverse effects after intravenous TNF treatment. In order to increase therapeutic index, TNF was tested in combination with other treatment modalities. In most combinations, TNF has proved to act synergistically. Combinations with other cytokines, such as interferons, IL-2, topoisomeraze targeted chemotherapeutics, radiotherapy, electrotherapy, and hyperthermia were also tested. Perspectives of TNF applications seem to be in local TNF treatment, aimed to increase its antitumor effectiveness and decrease side effects.*

Conclusions. *Additionally, most of these combined modality treatments require clinical testing. These directions may path the way for TNF into broader clinical use.*

Ključne besede: *tumorski nekrozni faktor; radiacijska terapija; kemoterapija*

Izvleček – Izhodišča. *Citokini vplivajo na mnoge biološke procese v organizmu, posebno na tiste, ki so udeleženi pri regulaciji rasti, aktivaciji in diferenciaciji celic. Tumorski nekrozni faktor (TNF) je bil prvotno odkrit kot molekula s protitumorsko aktivnostjo. V zadnjem času pa je vse pomembnejša njegova imunoregulatorna vloga v mreži citokinov. TNF je pomemben mediator pri imunskem odzivu in pri vnetnih reakcijah. Čeprav je selektivno citotoksičen za nekatere tumorske celice in vitro, ta njegova lastnost ni vedno izražena v protitumorski aktivnosti in vivo. Mnogi tumorji se odzivajo na zdravljenje s TNF, čeprav so njihove tumorske celice in vitro rezistentne na TNF. To nakazuje, da v organizmu TNF deluje predvsem posredno, z delovanjem na vaskulaturo tumorja in spodbujanjem imunskega odziva organizma. Čeprav je TNF zelo uspešen v protitumorskem delovanju na solidne tumorje eksperimentalnih živali, so rezultati kliničnih študij razočarali. Zelo malo je bilo objektivnih odgovorov na sistemsko terapijo s TNF, ki so jo spremljali hudi stranski učinki. Da bi izboljšali terapevtski indeks, je bilo narejenih več študij, kjer so kombinirali TNF z drugimi uveljavljenimi terapevtskimi pristopi. Pri mnogih kombinacijah je bil dokazan sinergističen protitumorski učinek. Znane so kombinirane terapije z drugimi citokini, topoizomerazno usmerjenimi kemoterapevtiki, radioterapijo, elektroterapijo in hipertermijo. Preizkuša se tudi lokalno zdravljenje s TNF, z namenom, da bi obranili protitumorski učinek in zmanjšali stranske učinke.*

Zaključki. *Večina naštetih kombiniranih terapij še ni bila preizkušena v kliniki, vendar je verjetno ravno v tem in v lokalnem zdravljenju perspektiva uporabe TNF v klinični praksi.*

Introduction

At the end of the 18th century, physicians observed regressions of the tumors in patients who had been through a severe infection. These observations led William B. Coley to experimentation with Gram-negative and Gram-positive bacteria preparations (Coley's toxins) in the treatment of a variety of cancer patients. Although this first attempt to use biological response modifier has to be seen in the context of that time, his well-documented cases of successful treatment of cancer patients are very impressive (1). This clinical testing led to fundamental, well-controlled research in animal systems. It was recognized that lipopolysacharides of bacterial cell walls, the so-called endotoxin, represent the active principle responsible for the regression of tumors. In 1975, Lloyd Old and co-workers demonstrated that endotoxin by itself was not directly involved in antitumor effects, but that it caused the induction of a factor in the serum of animals, which in turn was responsible for tumor necrosis (2). The factor was named tumor necrosis factor, or TNF. The biological activity of this factor can be determined at in vivo and in vitro levels. Application of TNF-containing serum in mice with transplantable, methylcholanthrene-induced fibrosarcoma leads to rapid hemorrhagic necrosis of the tumors (2). In vitro it was shown that TNF-containing serum was selectively cytotoxic to a number of malignant cells (2, 3). Further studies

indicated that TNF is produced primarily by macrophages (4). Later it was shown that not only macrophages, but also lymphocytes were able to synthesize TNF. This TNF synthesized by lymphocytes was named lymphotoxin. The relationship between TNF and lymphotoxin was not clear until both proteins were purified to homogeneity, primary structure determined, and their cDNA's isolated and expressed (5). These studies revealed similarities in the proteins and genes of the two molecules. The biological activities of the two molecules were found to be similar, and therefore the names TNF-α and TNF-β were applied to the monocyte and lymphocyte derived proteins, respectively.

TNF molecule

The TNF-α and TNF-β genes are single copy genes, closely linked within the cluster of major histocompatibility complex (MHC) genes. They are located on the short arm of human chromosome 6 (6) and murine chromosome 17 (7). The TNF-β gene is always 5' to the TNF-α genes in all the species examined so far (8). Genes for both TNF-α and TNF-β are each 3 kB long and consist of four exons and three introns (8). These similarities strongly suggest that the two genes have been derived from a common ancestral gene by gene duplication. More similarities were found in the region coding for mature protein (80–89%) than in the elements that code for the regulation and transcription (9).

The human TNF-α cDNA gene codes for a mature polypeptides of 157 amino acids, preceded by a 76 amino acid long pre-sequence (10). This pre-sequence is strongly conserved among different species, which indicates a specific, if not essential function (11). TNF-α molecule can exist also in unprocessed, membrane-bound form (12). There is evidence that this long pre-sequence serves to anchor the TNF-α precursor molecule in the plasma membrane (11). The portion of the transmembrane form of TNF-α molecule exposed to the outside of the cell not only serves as a precursor for released TNF-α, but can also bind itself to an adjacent cell and thereby engage in intercellular communication (12–14).

The native structure of TNF-α and TNF-β molecules are trimers with a total molecular mass of 52 kDa (15). The shape of the molecule resembles a triangular cone, in which each of the three subunits has a typical jelly roll-β structure. The structure quite remarkably resembles the arrangements of many viral capsids, especially Satellite Tabacco Necrosis Virus (STNV) capsid protein (11). The significance of this similarity is not understood, but raises the question of their convergent or divergent evolution.

The question is where on the molecule are the active sites? Studies with randomly obtained mutant molecules revealed that the active site must be located on the lower half of the triangular pyramid, in the groove between the subunits (11). This active site also corresponds to the receptor binding domain, and follows its three-dimensional symmetry.

TNF receptors

TNF receptors are present on many cell types, both normal and transformed, with only few exceptions, such as erythrocytes and unstimulated T-lymphocytes (11, 16). The number of TNF receptors (TNF-R) varies on the cells, from 200 up to 10,000 (17). Although they are prerequisite for biological effect, no correlation between the number of the TNF-R and the magnitude of the effect has been found (17). Protein purification and cDNA cloning studies identified two distinct receptors, the 55-kDa (TNF-R55 or TNF-RI) and the 75-kDa (TNF-R75 or TNF-RII) (18, 19) receptors. The extra-cellular portions of these receptors are similar not only to each other but also to the extra-cellular domains of the nerve growth factor (NGF) and several structurally related surface molecules (8). The intra-cellular portions of the receptors are different, there is no homology in this region (20). The lack of resemblance of the intracellular portion suggests that the two receptors activate different intracellular signalling pathways without any hints as to its function. The TNF-R55 seems to be present on most cell types, among others on epithelial cells and fibroblasts, while TNF-R75 is restricted to the cells of hematopoietic origin (8, 11). Some cells were found to express only one receptor, while most cells examined had both receptors, expressed in different proportions (20, 21).

Soluble TNF-binding proteins have been characterized in human serum and urine. They were originally identified as TNF inhibitory peptides, but are now known to be truncated portions of the extracellular domains of the two TNF-R (22, 23). The function of these TNF-binding peptides could be in regulating the bioactivity of TNF-α in the body. Soluble TNF-R may compete and inhibit TNF-α action on the cells, but on the other hand, by binding to TNF-α in dissociable form, it may affect the pharmacokinetics and stability of TNF-α. An interesting possibility is that soluble TNF-R, which are present in higher concentrations in tumor-bearing subjects, may affect the pharmacokinetics of TNF-α after the treatment. This was demonstrated on sarcoma- and melanoma-bearing mice treated with TNF-α. Comparison of TNF-α serum concentration profiles in tumor-bearing and healthy animals indicates that the absorption, distribution and/or metabolism in tumor-bearing animals differs significantly from those in healthy animals (24, 25).

TNF action on cells

TNF-α was originally an object of great interest because of its selective cytotoxicity to malignant cells (2). With recombinant TNF-α production, several human and animal tumor cell lines have been tested for TNF-α cytotoxicity (3, 26). Now it is known that TNF-α is cytotoxic to many cells, which have a wide range of susceptibility, from high to resistant (26, 27). The cytotoxicity of TNF-α can be increased many-fold by actinomycin D or cycloheximide (28). Major steps in the TNF-α-mediated cytotoxicity cascade include G protein-couple activation of phospholipases, generation of free radicals and damage to nuclear DNA by endonucleases (29). Cell membrane fluidity also increases after TNF-α treatment, and correlates with cytotoxicity (30). Such alterations can contribute to the cytotoxicity of TNF-α, or they are a step in cytolysis. But cell lysis is not the only way in which TNF-α can kill, in some cell types it can lead to apoptosis, a programmed cell death (31).

On the other hand, TNF-α can exert proliferative activity on a number of cell types, especially on fibroblasts (3, 32) (Tab. 1). Additionaly, on some transformed cells, the growth-enhancing activity of the TNF-α was demonstrated (33, 34). Biphasic effect on cell growth was observed; inhibition of cell growth in high TNF-α concentrations and stimulation of cell growth in low concentrations. Because blocking RNA or protein synthesis strongly enhances TNF-α cytotoxicity, it is often suggested that TNF-α itself induces synthesis of protective proteins, which interfere with generation of toxic products, or help to detoxify them (35).

In many cell types, TNF-α causes a release of arachidonic acid, which leads to secretion of PGE_2 and some other eicosanoids (36). Treatment of, for example, endothelial cells induces synthesis of platelet-derived growth factor (PDGF) (37). In neutrophils, TNF-α induces respiratory burst and degranulation, releasing elastase, lysozyme and other enzymes (38).

In many cells, TNF-α induces various sets of genes by transcriptional activation. This has been studied primarily on fibroblasts and endothelial cells. Gene products can be found in nucleus (c-fos, c-jun, c-myc) (39, 40), in mitochondia (Mn-superoxide dismutase) (11), in cytoplasm, on the cell membrane, or secreted in the medium. For example, in endothelial cells, the new antigens appearing on the membrane are the procoagulant factor (41),

Tab. 1. *Some biological activities mediated by TNF-α*

Cell type	Effect
Endothelial cells	Procoagulant activity Cytostatic/cytotoxic
Adipocytes	Differentiation Suppression of lipogenic enzymes
Fibroblasts	Production of collagenase and PGE2 Proliferation
Synovial cells	Production of collagenase and PGE2
Muscle cells	Inhibition of contractability Inhibition of myoblasts differentiation
Bone cells	
Osteoblasts	Growth inhibition Inhibition of collagen synthesis
Osteoclasts	Activation, proliferation
Cells of CNS	
Cells of hypothalamus	Production of PGE2
Astrocytes	Proliferation
Oligodendrocytes	Cytotoxicity
Cells of immune system	
Hematopoietic cells	Inhibition of colony formation
Monocytes/macrophages	Activation, chemotaxis
Neutrophils	Activation, chemotaxis
Eosinophils	Increased toxicity to pathogens
Tymocytes	Proliferation
T lymphocytes	Proliferation
B lymphocytes	Proliferation, differentiation
NK/LGL	Induction of LAK activity
Tumor cells	Cytostatic/cytotoxic
Virus infected cells	Inhibition of virus replication Cytotoxic

membrane-bound IL-1 (42), and enhancement of class I-HLA structures (43). Examples of secreted proteins are IL-6, GM-CSF, M-CSF and plasminogen activator inhibitor (PAI) (44).

Immunomodulatory effect of TNF

TNF-α is a pleiotropic molecule, which can display a series of effects in different cell types (Tab. 1). As a product of monocyte/macrophage lineage, TNF-α plays an important role in mechanisms augmenting the effector activities of the immune cells. Monocytes/macrophages, activated by various agents, can be rendered cytotoxic and kill other cells. Several mechanisms have been suggested, and according to one of the theories cytotoxicity is mediated by TNF-α (45), which has put TNF-α in the central position as an effector molecule in cytotoxicity.

In an autocrine fashion, TNF-α induces itself in monocytes, activates them and stimulates their cytotoxicity (46). TNF-α also induces the synthesis of interleukin-1 (IL-1) (47), expression of Fc receptors (48) and of Ia antigens (49) on macrophages.

The action of TNF-α on lymphocytes is displayed after initial stimulation, because resting T-lymphocytes appear to lack the TNF-R (50, 51). TNF-α enhances proliferation of T-lymphocytes in dose-dependent manner, modulating proliferation and differentiation of B-lymphocytes (51, 52). High concentrations of TNF-α induce T-lymphocytes to release IFN-γ, and TNF-α exerts synergistic effect with IL-2 in the generation of LAK cells (53).

Since macrophages play a central role in orchestrated immune response to stimuli, TNF-α molecule has also been put into the same position. Because of its pleiotropic action on different cells of the immune system, TNF-α has to be carefully regulated because of its adverse effects in the case of overproduction. Some of its antagonists have already been identified, but thus require further investigations, in order to solve the problems where overproduction of TNF-α induces pathological situations associated with several diseases.

The role of TNF in diverse pathological processes

Several cell-to-cell communications are crucial during the interaction and maintenance of specific foci of inflammation. Cytokines act as local mediators of cellular homeostasis and TNF-α plays a key role among them. One of the important events that occur during local immune inflammatory response is manifested by the effect of TNF-α on the endothelial cells. TNF-α has been shown to stimulate angiogenesis (54) and to alter endothelial cell responsiveness (55). TNF-α stimulates endothelial cells to produce GM-CSF, IL-1 and class I MHC molecules (44, 56). TNF-α and IL-1 promote accumulation of granulocytes at the site of inflammation, by enhancing the expression of surface adherence molecules (57). In addition, TNF-α induces respiratory burst, degranulation, phagocytosis and antibody-dependent cellular cytotoxicity of the neutrophils (58). TNF-α also increases the production of procoagulants and downregulates the production of trombomodulin, thus converting vascular endothelium to procoagulant surface (59). All these events lead to cell extravasation and activation, and cessation of blood flow leading to tissue necrosis.

Intervention at the inflammatory state, caused by high release of cytokines and TNF-α, can theoretically be blocked by substances or agents inhibiting the inflammation (i. e. antibiotics). The other ways may be to block the release of TNF-α, neutralize TNF-α or TNF-R, and thus prevent the inflammatory response from amplifying itself. In this direction some studies with TNF-α antibodies have been conducted. Protection from lethal effects of endotoxin was achieved (60).

Cachexia, the severe wasting, often accompanies chronic parasitic, bacterial or viral infections, and is all too often a major symptom in cancer patients. Animals which are cachectic have high triglyceride levels in circulation, presumably due to depressed lipoprotein lipase activity. A factor that not only mediates lipoprotein lipase inhibition in vivo (61) but also suppresses this enzyme in adipocytes in vitro was identified (62). This factor, which was named cachexin turned out to be identical to TNF-α (63).

Septic shock is usually a result of infection with Gram-negative bacteria or high doses of LPS. There is evidence that TNF-α plays a key role in septic shock. It was found that septic shock with fatal outcome significantly correlates with high levels of TNF-α in serum (64).

Elevated levels of TNF-α appear to be involved also in the processes associated with other pathologic conditions, including diseases of parasitic origin, allograft rejection, viral diseases and HIV infection (44, 65).

Mechanisms of TNF action on tumors

TNF-α was named after the rapid necrosis observed in transplantable, methylcholanthrene-induced sarcomas in mice (2). These tumors are atypical, and hardly a model for cancer in man. TNF-α treatment in mice induces rapid necrosis of the central portion of solid tumors within 48 hours. With high doses even curative results can be achieved, otherwise tumors regress for a certain period of time, and thereafter regrow again from the remaining viable tumor cells in the outer portion of the tumors (2, 66, 67). The fact that certain tumor cell lines can be in vitro resistant to TNF-α, whereas in vivo solid tumors originating from the same cell line are highly responsive to TNF-α treatment, came as a surprise (44, 68). This discrepancy between in vitro and in vivo antitumor effectiveness of TNF-α led to the conclusion that in vivo antitumor effects of TNF-α are host-mediated. Direct tumor cell cytotoxicity is less likely, because it is achieved only in high TNF-α concentrations. For the antitumor effectiveness of TNF-α, tumors must be well-vascularizied in order to induce hemorrhagic necrosis, because treatment of ascitic tumors or micro-metastases with TNF-α has

little or no antitumor effect (68–70) (Fig. 1). Since tumor destruction takes place only in vascularised neoplasms, the indirect action seems to be mediated by the efficacy of TNF-α on the vascular endothelium of the tumor circulation. Histopathological investigations have confirmed that tumor necrosis induced by TNF-α is due to circulatory disturbance associated with micro-vascular injury within the tumor, which is manifested by hyperemia and multiple fibrin thrombi at capillary level (71, 72). In some of the highly immunogenic tumors, the action of TNF-α is at least partly T-cell dependent (73). This was confirmed by the resistance of the cured mice to challenge by the same tumor cells (11), and by partial abolition of the response in immunosuppressed animals (70, 74). Moreover, the immune system is important, since tumor necrosis cannot be achieved in syngeneic nude mice (75, 76). Thus, in addition to direct cytotoxicity, the antitumor effect of TNF-α on animal experimental models may be two-fold, i.e. via effects on tumor vasculature, and by stimulation of a specific host immune antitumor response. How this concept correlates with antitumor effectiveness of TNF-α in humans, where tumors are poorly immunogenic, is not clear.

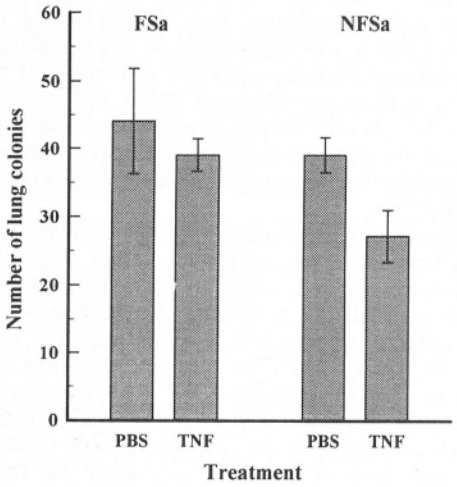

Fig. 1. *Effect of TNF-α treatment (4 × 10⁴ U/mouse, three times intravenously on days 4, 5 and 6) on FSa and NFSa tumor microcolonies in the lungs.*

The hemorrhagic necrosis is induced predominately in tumors, although damage to the vascular endothelium is observed in other organs, mainly in lung and gastrointestinal tract (77–79). Higher sensitivity of tumor endothelium can be explained on the basis of the physiological differences between mature vessels and those newly evoked within solid tumors. The newly formed vessels in tumors are usually thin-walled capillaries or sinusoids with little more than an endothelial lining, backed by a basement membrane (80). Additionally, higher proliferation rate of endothelium in solid tumors is one of the prominent differences between solid tumors and normal tissues (81). All these factors may be contributing to the differential susceptibility of the vascularization in solid tumors and normal tissues.

Because of many other adverse effects of TNF-α treatment, such as fever, hypotension, diffuse pulmonary inflammation, hemorrhagic lesions in the gastrointestinal tract and diffuse intravascular thrombosis (77–79), different routes of TNF-α application have been tested. The comparison of systemic and intratumoral or peritumoral application is especially interesting. TNF-α application into the tumor or into its immediate vicinity, has proved to be equally or, in some cases, even more effective than intravenous application (70, 74, 82, 83) (Fig. 2). This route of application usually induces less side effects, because of slower TNF-α elimination and lower serum concentrations (24, 25). Also, direct tumor cell cytotoxicity is a more likely antitumor mechanism, because higher TNF-α concentrations in the tumor are achievable. After the initial testing on animal models, intratumoral and peritumoral applications are becoming more relevant also for clinical application.

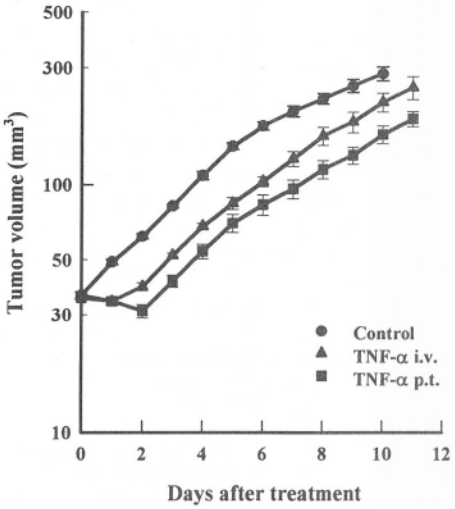

Fig. 2. *Antitumor effect of TNF-α on subcutaneously growing SA-1 tumors. Tumors were treated with 2 × 10⁵ U TNF-α intravenously or peritumorally.*

TNF in combined treatment with other treatment modalities

Because tumor cures can seldom be achieved with TNF-α treatment, researchers are looking for new treatment combinations. Their aim is to increase the therapeutic index by decreasing the TNF-α toxicity or by increasing its effectiveness. Therefore, attempts have been made to combine TNF-α with several other biological response modifiers, chemotherapeutic drugs, radiotherapy, hyperthermia, electrotherapy and others.

Several in vitro studies have shown interaction between interferons and TNF-α. IFN-γ and IFN-α up-regulate TNF-R on tumor cells, which influences other biological activities of TNF-α (84). This is usually reflected also in synergistic cytotoxicity of TNF-α and interferons to tumor cells. Besides interaction of TNF-α with IFN-α (27, 85) (Fig. 3), most studies have been done with IFN-γ (3, 26, 27, 33, 86), which has also shown the best results. At in vivo level, several studies have dealt with the same treatment combinations, and have confirmed that interferons, with appropriate protocol, interact with TNF-α antitumor effects (87–89). Also elimination of the tumors and complete cures can be achieved (11). Synergistic antitumor effect has been noted also in combination of TNF-α with IL-1, IL-2, and IL-4 (89, 90). Potentiation of TNF-α antitumor activity was tested also with desmuramyl dipeptide analogs LK-409 and LK-410 (91). Muramyl dipeptides are the minimal structures of Mycobacterium or Freund's adjuvant possessing immunoadjuvant activity. Combined treatment of fibrosarcoma tumors with TNF-α and LK-409 or LK-410 augments the antitumor effect of TNF-α. This demonstrates that many other immunological substances, such as muramyl dipeptide analogs, have the potential to increase the antitumor activity of TNF-α.

Combined treatment with TNF-α and chemotherapy demonstrated, in in vitro and in vivo studies, a potentiation of TNF-α antitumor activity. TNF-α cytotoxicity has been shown by several authors to be augmented by actinomycin D in TNF-α assay, involving L 929 cells and HL 60 cells (92, 93). The cells, normally resistant to TNF-α, were made sensitive by actinomycin D (93). Synergistic cytotoxic effect was shown with standard antitumor cancer drugs such as 5-FU, adriamycin and bleomycin (94, 95). Especially promising are combinations with chemotherapeutic drugs, which are primarily targeted to DNA topoisomerase II (96). Drugs, such

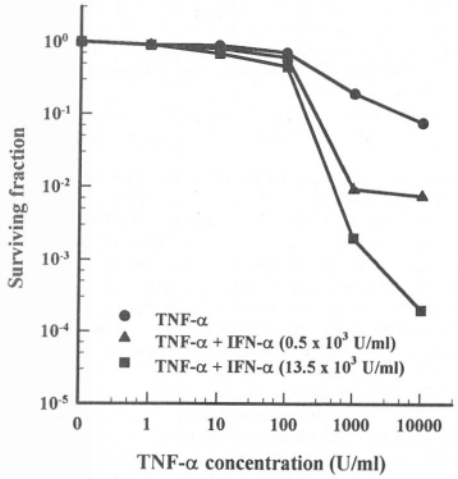

Fig. 3. *Synergistic cytotoxic effect of TNF-α and IFN-α on A-375-M human malignant melanoma cell line. Cells were grown in the presence of either of cytokines alone or in combination of both. Survival curves of combined treatment are normalized for the cytotoxicity of IFN-α (Sf in 0.5 × 10³ U/ml IFN-α was 0.65 and in 13.5 × 10³ U/ml IFN-α 0.15).*

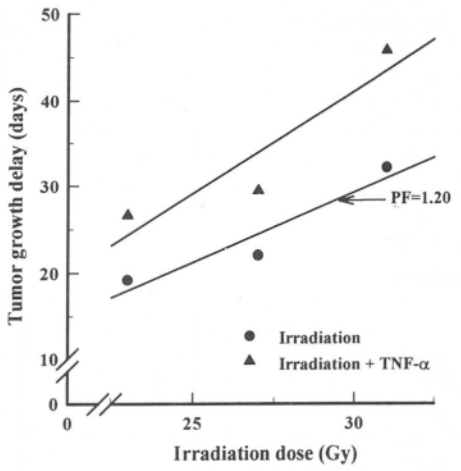

Fig. 4. *Tumor growth delay of intramuscular MCA-K tumors after different doses of irradiation in combination with TNF-α. Tumor growth delay was measured at 300 mm³ tumor volume, and the potentiating factor at 28 days. The results are normalized for the tumor growth in control groups, including TNF-α treated animals.*

Drugs, such as adriamycin, doxorubicin, teniposide and etoposide, are all topoisomeraze targeted and they are also widely used chemotherapeutic drugs. Besides, TNF-α alone can be effective to tumor cells resistant to cytotoxic chemotherapeutic drugs, especially if TNF-α is combined with IFN-γ (97). Therefore, combinations of TNF-α with chemotherapeutic drugs seem to be promising and provide a sound basis for clinical trials.

TNF-α was also investigated for its ability to increase the response of murine tumors to ionizing radiation. Treatment with TNF-α can augment radioresponse of murine tumors exposed to single or fractionated radiation (74, 98). The therapeutic index is increased when treatment with TNF-α follows irradiation of solid tumors. The effect of treatment with both agents combined is greater than the additive effect of the individual treatments (Fig. 4). Furthermore, TNF-α significantly increases tumor radiocurability. The augmented radioresponse of the tumors is not a result of increased radiosensitivity of the tumor cells, but is mediated through the antitumor mechanisms of the organism, either immunological or non-immunological. TNF-α is also effective in reducing damaging effect of ionizing radiation on bone-marrow progenitor cells. This can increase therapeutic advantage of TNF-radiotherapy combination. These experiments suggest that TNF in combination with radiotherapy may be beneficial for the treatment of cancer patients.

TNF-α cytotoxicity is increased also at higher temperatures. A synergistic increase in cytotoxic effect of TNF-α by hyperthermia was demonstrated both in vitro and in vivo. The results show that the hyperthermia augments lysosomal enzyme activation and induction of hydroxyl radical production by TNF-α (99). The greatest enhancement occurs with the simultaneous administration of TNF-α and hyperthermia. Hyperthermia was shown to be more cytotoxic under conditions of hypoxia and low pH, which has been shown to be the result of TNF-α treatment in vivo. Therefore the results of TNF-α and hyperthermia produce an enhanced antitumor effect against a transplantable fibrosarcoma in mice (100).

Combination of TNF-α with electrotherapy has also been tested on tumor models. Electrotherapy with direct current can effectively control local tumor growth and can be therefore considered in cancer treatment (101). The antitumor effect of anodic or cathodic electric current delivered through Pt/Ir electrodes is similar, though seldom resulting in tumor cures. Since application of electrotherapy can be envisioned in local/regional treatment of solid tumors, combined modality treatment with other cytotoxic treatments or biological response modifiers is interesting (102–104). Combination of both therapies, TNF-α and electrotherapy results in effective tumor control with additive antitumor effect, regardless whether TNF-α is injected before or after electrotherapy (105). More than an additive antitumor effect is achieved when TNF-α in the same cumulative dose is split into a low priming dose one hour before electrotherapy and a higher dose 24 hours thereafter. As a result, a high percentage of the animals can be cured, compared to non-curative effect of single treatments.

All these treatment combinations are still in experimental, preclinical phase. Although they have proved to be effective, many of them await its clinical applications.

The prospects for therapy with TNF

The first clinical trials which have used TNF-α as a chemotherapeutic agent were relatively disappointing. Preliminary reports on the clinical administration of recombinant TNF-α do not indicate significant therapeutic effect. Some partial responses have been seen in both hematological and solid tumors, but were all short lived (106). Dose-limiting side effects were fever, chills, rigor, fatigue, diarrhea, headache, nausea and vomiting, severe hypotension and fluid retention, most likely appearing as a consequence of a capillary-leak syndrome similar to that described for IL-2 (106). But recently some encouraging results were obtained in clinical trials with high-dose TNF-α treatment in isolated limb perfusion (107), in the treatment of ascites (108), or other forms of loco-regional treatment (106). Treatment results are often better in combination with other cytokines (IFN-γ, IL-2) (109), therefore these combinations may contribute to TNF-α's broader use in clinics. Also Coley's toxin must have induced a cocktail of cytokines, not just TNF-α, therefore we have to search for an adequate combination of different cytokines to obtain good clinical results.

Elevated levels of TNF-α were detected in several pathological conditions, including cancer (44, 110). Besides TNF-α, soluble TNF-R has also been determined in processes associated with malignant growth. Serum concentrations of soluble TNF-R in cancer patients often correlate with the stage of the disease (111, 112). Its role is probably to block TNF-α bioactivity and may therefore have bearing on edogenously formed TNF-α in cancer

patients, as well as on tumor development. Some believe that the determination of soluble TNF-R in serum of cancer patients may have implications in early detection and diagnostics, follow-up, and prognosis of cancer. There is also ongoing search for TNF-α analogs, TNF-α molecules with preserved biological activity but less side effects. Some reports already describe TNF-α analogs which have proved to be equally effective as TNF-α molecule in animal studies, but had considerably less side effects (113, 114).

Conclusion

With a more profound understanding of relationships between cytokines, the use of cytokine combinations in tumor therapy will become more effective. Only then it will be possible to bring the cytokine cocktail closer to the effects that will result in tumor regression. Combined modality treatments with other cytotoxic treatments such as chemotherapy and radiotherapy may give TNF its place in regional cancer treatment. Besides the role TNF plays in cancer therapy, the determination of its soluble receptors in serum may provide additional information to biology of cancer, and add information to cancer diagnosis, prognosis and follow-up. In other pathological processes, TNF plays undoubtedly a very important role, therefore, better understanding of its interaction in cytokine network will help to explain problems related to the control of those diseases.

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References

1. Coley WB. The treatment of the inoperable sarcoma by bacterial toxins the mixed toxins of the Streptococcus erysipelatis and the Bacillus prodigiosus. Proc R Soc Med Surg Sec 1909; 3: 1–48.
2. Carswell EA, Old LJ, Kassel RL, Green KS, Fiore N, Williamson B. An endotoxin-induced serum factor that causes necrosis of tumors. Proc Nat Acad Sci 1975; 72: 3666–70.
3. Sugarman BJ, Aggarwal BB, Hass PE, Figari IS, Palladino MA, Shepard HM. Recombinant human tumor necrosis factor-α: Effects on proliferation of normal and transformed cells in vitro. Science 1985; 230: 943–5.
4. Matthews N. Tumor necrosis factor from the rabbit. II. Production by monocytes. Br J Cancer 1978; 38: 310–5.
5. Pennica D, Nedwin GE, Hayflick JS, Seeburg PJ, Derynck R, Palladino MA, Kohr WJ, Aggarwal BB, Goeddel DV. Human tumor necrosis factor: Precursor structure, expression and homology to lymphotoxin. Nature 1984; 313: 724–9.
6. Spies T, Morton CC, Nedospasov SA, Fiers W, Pious D, Strominger JL. Genes for tumor necrosis factor α and β are linked to the human major histocompatibility complex. Proc Natl Acad Sci USA 1986; 83: 8699–702.
7. Müller U, Jongeneel CV, Nedospasov SA, Lindahl KF, Steinmetz M. Tumor necrosis factor and lymphotoxin genes map close to H-2D in the mouse histocompatibility complex. Nature 1987; 325: 265–7.
8. Vilček J, Lee TH. Tumor necrosis factor, new insights into the molecular mechanisms of its multiple actions. J Biol Chem 1991; 266: 7313–6.
9. Spies T, Blanck G, Bresnahan M, Sands J, Strominger JL. A new cluster of genes within the human major histocompatibility complex. Science 1989; 243: 214–7.
10. Wang AM, Creasey AA, Ladner MB, Lin LS, Strickler J, Van Arsdell JN, Yamamoto R, Mark DF. Molecular cloning of the complementary DNA for human tumor necrosis factor. Science 1985; 228: 149–54.
11. Fiers W. Tumor necrosis factor. Characterization at the molecular, cellular and in vivo level. FEBS 1991; 285: 199–212.
12. Kriegler M, Perez C, DeFay K, Albert I, Lu SD. A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: Ramification for the complex physiology of TNF. Cell 1988; 53: 45–53.
13. Decker T, Lohmann-Matthes ML, Gifford GE. Cell-associated tumor necrosis factor (TNF) as a killing mechanism of activated cytotoxic macrophages. J Immunol, 1987; 138: 957–62.
14. Feinman R, Henriksen-DeStefano D, Tsujimoto M, Vilček J. Tumor necrosis factor is an important mediator of tumor cell killing by human monocytes. J Immunol 1987; 138: 635–40.
15. Jones EY, Stuart DI, Walker NPC. Structure of tumor necrosis factor. Nature 1989; 338: 225–8.
16. Aggarwal BB, Eesalu TE, Haas PE. Characterization of receptors for human tumor necrosis factor and their regulation by γ-interferon. Nature 1985; 318: 665–7.
17. Kull FC, Jacobs S, Cuatrecasas. Cellular receptor for ^{125}I-labelled tumor necrosis factor: Specific binding, affinity labelling, and relationship to sensitivity. Proc Natl Acad Sci USA 1985; 82: 5756–60.
18. Hohmann HP, Remy R, Brockhaus M, Loon APGM. Two different cell types have different major receptors for human tumor necrosis factor (TNF-α). J Biol Chem 1989; 264: 1427–30.
19. Brockhaus M, Schoenfeld HJ, Schlaeger EJ, Hunziker W, Leslauer W, Loetscher H. Identification of two types of tumor necrosis factor receptors on human cell lines by monoclonal antibodies. Proc Natl Acad Sci USA 1990; 87: 3127–31.
20. Dembic Z, Loetscher H, Gubler U, Pan YC, Lahm HW, Gentz R, Brockhaus M, Lesslauer W. Two human TNF receptors have similar extracellular, but distinct intracellular, domain sequences. Cytokine 1990; 2: 231–7.
21. Smith CA, Davis T, Anderson D, Solam L, Beckmann MP, Jerzy R, Dower SK, Cosman D, Goodwin RG. The receptor for tumor necrosis factor defines an unusual family of cellular and viral proteins. Science 1990; 248: 1019–23.
22. Seckinger P, Isaaz S, Dayer JM. Purification and biological characterization of a specific tumor necrosis factor α inhibitor. J Biol Chem 1989; 264: 11966–73.
23. Seckinger P, Zhang JH, Hauptmann B, Dayer JM. Characterization of a tumor necrosis factor α (TNF-α) inhibitor: Evidence of immunological cross reactivity with the TNF receptor. Proc Natl Acad Sci USA 1990; 87: 5188–92.
24. Kus B, Serša G, Novaković S, Štalc A. Serum TNF-α levels in melanoma-bearing and healthy mice. Radiol Oncol 1992; 26: 223–7.
25. Kus B, Serša G, Novaković S, Urbančič J, Štalc A. Modification of TNF-α pharmacokinetics in SA-1 tumor-bearing mice. Int J Cancer 1993; 55: 110–4.
26. Fransen L, Van Der Heyden J, Ruysschaert R, Fiers W. Recombinant tumor necrosis factor: Its effect and its synergism with interferon-γ on a variety of normal and transformed human cell lines. Eur J Cancer Clin Oncol 1986; 22: 419–26.
27. Williamson BD, Carswell EA, Rubin BY, Prendergast JS, Old LJ. Human tumor necrosis factor produced by human B-cell lines: Synergistic cytotoxic interaction with human interferon. Proc Natl Acad Sci USA 1983; 80: 5397–401.
28. Ruff MR, Gifford GE. Tumor necrosis factor. In: Pick E ed. Lymphokines. Vol 2. New York: Academic Press Inc., 1981: 235–72.
29. Larrick JW, Wright SC. Cytotoxic mechanisms of tumor necrosis factor-α FASEB J 1990; 4: 3215–23.
30. Štalc A, Šentjurc M, Serša G, Novaković S. The influence of TNF on the membrane fluidity of tumor cells. Cancer Letters 1992; 65: 183–7.
31. Bellomo G, Perotti M, Taddei F, Mirabelli F, Finardi G, Nicotera P, Orrenius S. Tumor necrosis factor α induces apoptosis in mammary adenocarcinoma cells by an increase in intracellular free Ca^{2+} concentration and DNA fragmentation. Cancer Res 1992; 52: 1342–6.
32. Vilček J, Palombella VJ, Henriksen-DeStefano, Swenson C, Feinman R, Hirai M, Tsujimoto M. Fibroblast enhancing activity of tumor necrosis factor and its relationship to other polypeptide growth factors. J Exp Med 1986; 163: 632–43.
33. Lewis GD, Aggarwal BB, Eessalu TE, Sugarman BJ, Shepard HM. Modulation of the growth of transformed cells by human tumor necrosis factor-α and interferon-γ. Cancer Res 1987; 47: 5382–5.
34. Goillot E, Combaret V, Ladenstein R, Baubet D, Blay JY, Philip T, Favrot M. Tumor necrosis factor as an autocrine growth factor for neuroblastoma. Cancer Res 1992; 52: 3194–200.
35. Kirstein M, Baglioni C. Tumor necrosis factor induces synthesis of two proteins in human fibroblasts. J Biol Chem 1986; 261: 9565–7.
36. Dayer BJM, Beutler B, Cerami A. Cachectin/tumor necrosis factor stimulates collagenase and prostaglandin E_2 production by human synovial cells and dermal fibroblasts. J Exp Med 1985; 162: 2163–8.
37. Hajjar KA, Hajjar DP, Silverstein RL, Nachman RL. Tumor necrosis factor-mediated release of platelet-derived growth factor from cultured endothelial cells. J Exp Med 1987; 166: 235–45.
38. Shalaby MR, Aggarwal BB, Rinderknecht E, Sevedersky LP, Finkle BS, Palladino MA. Activation of human polymorphonuclear neutrophil functions by interferon-γ and tumor necrosis factors. J Immunol 1985; 135: 2069–73.
39. Lin JX, Vilček J. Tumor necrosis factor and interleukin-1 cause a rapid and transient stimulation of c-fos and c-myc mRNA levels in human fibroblasts. J Biol Chem 1987; 262: 11908–13.
40. Brenner DA, O'Hara M, Angel P, Chojkier M, Karin M. Prolonged activation of jun and collagenase genes by tumor necrosis factor-α. Nature 1989; 337: 661–3.
41. Bevilaqua MP, Pober JS, Majeau GR, Fiers W, Cotran RS, Gimbrone MA. Recombinant tumor necrosis factor induces procoagulant activity in cultured human vascular endothelium: Characterization and comparison with the action of interleukin 1. Proc Natl Acad Sci USA 1986; 83: 4533–7.
42. Locksley RM, Heinzel FP, Shepard HM, Agosti J, Eessalu TE, Aggarwal BB, Harlan JM. Tumor necrosis factor α and β differ in their capacities to generate interleukin 1 release from human endothelial cells. J Immunol 1987; 139: 1891–5.
43. Collins T, Lapierre LA, Fiers W, Strominger JL, Pober JS. Recombinant human tumor necrosis factor increases mRNA levels and surface expression of HLA-A,B antigens in vascular endothelial cells and dermal fibroblasts in vitro. Proc Natl Acad Sci USA 1986; 83: 446–50.
44. Jäättelä M. Biology of disease. Biological activities and mechanisms of action of tumor necrosis factor-α/cachectin. Lab Invest 1991; 64: 724–42.
45. Decker T, Lohman-Matthes ML, Gifford GE. Cell-associated tumor necrosis factor (TNF) as a killing mechanism of activated cytotoxic macrophages. J Immunol 1987; 138: 957–62.
46. Mace KF, Ehrke MJ, Hori K, Maccubbin DL, Mihich E. Role of tumor necrosis factor in macrophage activation and tumoricidal activity. Cancer Res 1988; 48: 5427–32.

47. Burchett SK, Weaver WM, Westall JA, Larsen A, Kronheim S, Wilson CB. Regulation of tumor necrosis factor/cachectin and IL-1 secretion in human mononuclear phagocytes. J Immunol 1988; 140: 3473–81.
48. Hoffman M, Weiberg JB. Tumor necrosis factor alpha induces hydrogen peroxide production and Fc receptor expression, but not increased Ia antigen expression by peritoneal macrophages. J Leuk Biol 1987; 42: 704–8.
49. Chang RJ, Lee SH. Effects of interferon-gamma and tumor necrosis factor-alpha on the expression of an Ia antigen on a murine macrophage line. J Immunol 1986; 137: 2853–5.
50. Dinarello CA, Cannon JG, Wolff SM. Tumor necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin-1. J Exp Med 1986; 163: 1433–50.
51. Scheurich P, Thoma B, Ücer U, Pfizenmeier K. Immunoregulatory activity of recombinant human tumor necrosis factor (TNF-α): induction of TNF receptors on human T cells and TNF-α mediated enhancement of T cell responses. J Immunol 1987; 138: 1786–90.
52. Jelinek DF, Lipsky PE. Enhancement of human B cell proliferation and differentiation by tumor necrosis factor-α and interleukin-1. J Immunol 1987; 139: 2970–6.
53. Semenzato G. Tumor necrosis factor: a cytokine with multiple biological activities. Br J Cancer 1990; 61: 354–61.
54. Leibovich SJ, Polverini PJ, Shepard HM, Wieseman DM, Shively V, Nuseir N. Macrophage-induced angiogenesis is mediated by tumor necrosis factor-α. Nature 1987; 329: 630–2.
55. Nawroth PP, Stern DM. Modulation of endothelial cell hemostatic properties by tumor necrosis factor. J Exp Med 1986; 163: 740–5.
56. Broudy VC, Kaushansky K, Segal GM, Harlan JM. Tumor necrosis factor type α stimulates human endothelial cells to produce granulocyte/macrophage colony-stimulating factor. Proc Natl Acad Sci USA 1986; 83: 7467–71.
57. Gamble JR, Harlan JM, Klebanoff SJ, Vadas MA. Stimulation of adherence of neutrophils to umbilical vein endothelium by human recombinant tumor necrosis factor. Proc Natl Acad Sci USA 1985; 82: 8667–71.
58. Shalaby MR, Aggarwal BB, Rinderknecht E, Svedersky LP, Finkle BS, Palladino AM. Activation of human polymorphonuclear neutrophil functions by interferon-γ and tumor necrosis factors. J Immunol 1985; 135: 2069–73.
59. Stern D, Nawroth P, Handley D, Kisiel W. An endothelial cell-dependent pathway of coagulation. Proc Natl Acad Sci USA 1985; 82: 2523–7.
60. Beutler B, Milsark IW, Cerami AC. Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. Science 1985; 229: 869–72.
61. Stovroff MC, Fraker DL, Swedenborg JA, Norton JA. Cachectin/tumor necrosis factor: A possible mediator of cancer anorexia in the rat. Cancer Res 1988; 48: 4567–72.
62. Torti FM, Dieckmann B, Beutler B, Cerami A, Ringold GM. A macrophage factor inhibits adipocyte gene expression: an in vitro model for cachexia. Science 1985; 229: 867–9.
63. Beutler B, Greenwald D, Hulmes JD, Chang M, Pan YCE, Mathison J, Ulvetich R, Cerami A. Identity of tumor necrosis factor and the macrophage secreted factor cachectin. Nature 1985; 316: 552–5.
64. Bone RC. The pathogenesis of sepsis. Ann Int Med 1991; 115: 457–69.
65. Rook GAW, Taverne J, Playfair JHL. Evaluation of TNF as antiviral, antibacterial and antiparasitic agent. Biotherapy 1991; 3: 167–75.
66. Creasey AA, Reynolds MT, Liard W. Cures and partial regression of murine and human tumors by recombinant human tumor necrosis factor. Cancer Res 1986; 46: 5687–90.
67. Kawai T, Satomi N, Sato N, Sakurai A, Haranaka K, Goto T, Suzuki. Effects of tumor necrosis factor (TNF) on transplanted tumors induced by methylcholanthrene in mice. Virchow Arch (Cell Pathol) 1987; 52: 489–500.
68. Manda T, Shimomura K, Mukumoto S, Kobayashi K, Mizota T, Hirai O, Matsumoto S, Oku T, Nishigaki F, Mori J, Kikuchi H. Recombinant human tumor necrosis factor-α: evidence of an indirect mode of antitumor activity. Cancer Res 1987; 47: 3707–11.
69. Chun M, Hoffmann MK. Combination immunotherapy of cancer in a mouse model: synergism between tumor necrosis factor and other defense systems. Cancer Res 1987; 47: 115–8.
70. Havell EA, Fiers W, North RJ. The antitumor function of tumor necrosis factor. I. Therapeutic action of TNF against an established murine sarcoma is indirect, immunologically dependent, and limited by severe toxicity. J Exp Med 1988; 167: 1067–85.
71. Watanabe N, Niitsu Y, Umeno H, Kuriyama H, Neda H, Yamauchi N, Maeda M, Urushizaki I. Toxic effect of tumor necrosis factor on tumor vasculature in mice. Cancer Res 1988; 48: 2179–83.
72. Kallinowski F, Schaefer C, Tyler G, Vaupel P. In vivo targets of recombinant human tumor necrosis factor-α: blood flow, oxygen consumption and growth of isotransplanted rat tumors. Br J Cancer 1989; 60: 555–60.
73. Palladino MA Jr., Shalaby RM, Kramer SM. Characterization of the antitumor activities of human tumor necrosis factor alpha in the comparison with other cytokines: induction of tumor-specific immunity. J Immunol 1987; 138: 4023–32.
74. Seršа G, Willingham V, Milas L. Anti-tumor effects of tumor necrosis factor alone or combined with radiotherapy. Int J Cancer 1988; 42: 129–34.
75. Balkwill FR, Lee A, Aldam G, Moodie E, Thomas JA, Tavernier J, Fiers W. Human tumor xenografts treated with recombinant human tumor necrosis factor alone or in combination with interferons. Cancer Res 1986; 46: 3990–3.
76. Haranaka K, Satomi N, Sakurai A. Antitumor activity of murine tumor necrosis factor (TNF) against transplanted murine tumors and heterotransplanted human tumors in nude mice. Int J Cancer 1984; 34: 263–7.
77. Beutler BA, Milsark IW, Cerami A. Cachectin/tumor necrosis factor: production, distribution, and metabolic fate in vivo. J Immunol 1985; 135: 3972–7.
78. Tracey KJ, Beutler B, Lowry SF, Merryweather J, Wolpe S, Milsark IW, Hariri RJ, Fahev TJ, Zentella A, Albert JD, Shires GT, Cerami A. Shock and tissue injury induced by recombinant human cachectin. Science 1986; 234: 470–2.
79. Krosnick JA, McIntosh JK, Mule JJ, Rosenberg SA. Studies of mechanisms of toxicity of the administration of recombinant tumor necrosis factor α in normal and tumor-bearing mice. Cancer Immunol Immunother 1989; 30: 133–8.
80. Denekamp J. Vascular endothelium as the vulnerable element in tumours. Acta Radiol Oncol 1984; 23: 217–25.
81. Denekamp J. Vascular attack as a therapeutic strategy for cancer. Cancer Metast Rev 1990; 9: 267–82.
82. Sohmura Y, Nakata K, Yoshida H, Kashimoto S, Matsui Y, Furuichi. Recombinant human tumor necrosis factor-α. Antitumor effect on murine and human tumors transplanted in mice. Int J Immunopharmac 1986; 8: 357–68.
83. Serša G, Novaković S, Štalc A. Tumor mass is a critical factor in peritumoral treatment with tumor necrosis factor. Period Biol 1992; 94: 35–40.
84. Tsujimoto M, Yip YK, Vilček J. Interferon-γ enhances expression of cellular receptors for tumor necrosis factor. J Immunol 1986; 136: 2442–4.
85. Serša G, Milas L, Willingham V, Plesničar S. Synergism in cytotoxic action between tumor necrosis factor alpha and interferon alpha. Period Biol 1989; 91: 309–13.
86. Price G, Brenner MK, Prentice HG, Hoffbrand AV, Newland AC. Cytotoxic effect of tumor necrosis factor and gamma-interferon on acute myeloid leukacmia blasts. Br J Cancer 1987; 55: 287–90.
87. McIntosh JK, Mule JJ, Kosnick JA, Rosenberg SA. Combination cytokine immunotherapy with tumor necrosis factor α, interleukin-2, and α-interferon and its synergistic antitumor effects in mice. Cancer Res 1989; 49: 1408–14.
88. Balkwill FR, Lee AL, Aldam G, Moodie E, Thomas JA, Tavernier J, Fiers W. Human tumor xenografts treated with recombinant human tumor necrosis factor alone or in combination with interferons. Cancer Res 1986; 46: 3990–3.
89. Nio Y, Shiraishi T, Tsubono M, Morimoto H, Tseng CC, Kawabata K, Masai Y, Tun T, Fukumoto M, Tobe T. In vivo effects of human recombinant tumor necrosis factor alone and in combination with other biological response modifiers in human digestive organ cancer xenografts transplanted in nude mice. Biotherapy 1991; 3: 337–44.
90. Totpal K, Aggarwal BB. Interleukin 4 potentiates the antiproliferative effects of tumor necrosis factor on various tumor cell lines. Cancer Res 1991; 51: 4266–70.
91. Serša G, Novaković S, Štalc A. Antitumor effect of recombinant human tumor necrosis factor-α analog combined with desmuramyl dipeptides LK-409 or LK 410 on sarcoma in mice. Mol Biother 1992; 4: 188–92.
92. Flick DA, Gifford GE. Comparison of in vitro cell cytotoxic assays for tumor necrosis factor. J Immunol Methods 1984; 68: 167–75.
93. Darzynkiewicz Z, Carter SP, Old LJ. Effect of recombinant tumor necrosis factor on HL 60 cells: Cell cycle specificity and synergism with actinomycin D. J Cell Physiol 1987; 130: 328–35.
94. Peng LY, Liu R, Salmon S. Combinations of tumor necrosis factor (TNF) and anticancer drugs exhibit synergistic effects against human colon cancer cell lines. Proc Am Assoc Cancer Res 1987; 28: 383–6.
95. Krosnick JA, Mule JJ, McIntosh JK, Rosenberg SA. Augmentation of antitumor efficacy by the combination of recombinant tumor necrosis factor and chemotherapeutic agents in vivo. Cancer Res 1989; 49: 3729–33.
96. Alexander RB, Nelson WG, Coffey DS. Synergistic enhancement by tumor necrosis factor of in vitro cytotoxicity from chemotherapeutic drugs targeted at DNA topoisomerase II. Cancer Res 1987; 47: 2403–6.
97. Soranzo C, Perego P, Zunino F. Effect of tumor necrosis factor on human tumor cell lines sensitive and resistant to cytotoxic drugs, and its interaction with chemotherapeutic agents. Anti-Cancer Drugs 1990; 1: 157–63.
98. Nishiguchi I, Willingham V, Milas L. Tumor necrosis factor as an adjunct to fractionated radiotherapy in the treatment of murine tumors. Int J Radiat Oncol Biol Phys 1990; 18: 555–8.
99. Yamauchi N, Watanabe N, Maeda M, Okamoto T, Sasaki H, Tsuji N, Tsuji Y, Umeno H, Akijama S, Niitsu Y. Mechanisms of synergistic cytotoxic effect between tumor necrosis factor and hyperthermia. Jpn J Cancer Res 1992; 83: 540–5.
100. Watanabe N, Niitsu Y, Umeno H, Sone H, Neda H, Yamauchi N, Maeda M, Urushizaki I. Synergistic cytotoxic and antitumor effects of recombinant human tumor necrosis factor and hyperthermia. Cancer Res 1988; 48: 650–3.
101. Serša G, Miklavčič D. The feasability of low level direct current electrotherapy for regional cancer treatment. Reg Cancer Treat 1993; 6: 31–5.
102. Serša G, Novaković S, Miklavčič D. Potentiation of bleomycin antitumor effectiveness by electrotherapy. Cancer Letters 1993; 69: 81–4.
103. Serša G, Miklavčič D, Batista U, Novaković S, Bobanović F, Vodovnik L. Antitumor effect of electrotherapy alone or in combination with interleukin-2 in mice with sarcoma and melanoma tumors. Anti-Cancer Drugs 1992; 3: 253–60.
104. Serša G, Miklavčič D. Inhibition of SA-1 tumor growth in mice by human leukocyte interferon alpha combined with low level direct current. Mol Biother 1990; 2: 165–8.
105. Serša G, Golouh R, Miklavčič D. Anti-tumor effect of tumor necrosis factor combined with electrotherapy on mouse sarcoma. Anti-Cancer Drugs 1994; 5: 69–74.
106. Taguchi T, Sohmura Y. Clinical studies with TNF. Biotherapy 1991; 3: 177–86.
107. Lienard D, Ewalenko P, Delmotte JJ, Renard N, Lejeune FJ. High-dose recombinant tumor necrosis factor alpha in combination with interferon gamma

and melphalan in isolation perfusion of the limbs for melanoma and sarcoma. J Clin Oncol 1992; 10: 52–60.
108. Räth U, Kaufmann M, Schmid H, Hofmann J, Wiedermann B, Kist A, Kempani J, Schlick E, Bastert G, Kommerell B, Männel D. Effect of intraperitoneal recombinant human tumor necrosis factor alpha on malignant ascites. Eur J Cancer 1991; 27: 121–5.
109. Yang SC, Grimm EA, Parkinson DR, Carinhas J, Fry KD, Rodriguez AM, Licciardello J, Owen-Schaub LB, Hong WK, Roth JA. Clinical and immunomodulatory effect of combination immunotherapy with low-dose interleukin-2 and tumor necrosis factor-α in patients with advanced non small cell lung cancer: A phase I trial. Cancer Res 1991; 51: 3669–76.
110. Chopra IJ, Sakane S, Teco GNC. A study of the serum concentrations of tumor necrosis factor-α in tyroidal and nonthyroidal illnesses. J Clin Endocrinol Metab 1991; 72: 1113–6.
111. Aderka D, Engelmann H, Hornik V, Skornick Y, Levo Y, Wallach D, Kushtai G. Increased serum levels of soluble receptors for tumor necrosis factor in cancer patients. Cancer Res 1991; 51: 5602–7.
112. Digel W, Porzsolt F, Schmidt M, Hermann F, Lessiauer W, Brockhaus M. High levels of circulating soluble receptors for tumor necrosis factor in hairy cell leukemia and type B chronic lymphocytic leukemia. J Clin Invest 1992; 89: 1690–3.
113. Gatanaga T, Noguchi K, Tanabe Y, Inagawa H, Soma GI, Mizuno D. Antitumor effect of systemic administration of novel recombinant tumor necrosis factor (rTNF-S) with less toxicity than conventional rTNF-α in vivo. J Biol Response Mod 1989; 4: 278–86.
114. Van Ostade X, Vandenabeele P, Everaerdt B, Loetscher H, Gentz R, Brockhaus M, Lessiauer W, Tavernier J, Brouckhaert P, Fiers W. Human TNF mutants with selective activity on p55 receptor. Nature 1993; 361: 266–9.

Professional article/Strokovni prispevek

PRODUCTION OF RECOMBINANT HUMAN TUMOR NECROSIS FACTOR α IN TWO DIFFERENT ESCHERICHIA COLI EXPRESSION SYSTEMS

PRODUKCIJA REKOMBINANTNEGA HUMANEGA FAKTORJA TUMORSKE NEKROZE α Z DVEMA EKSPRESIJSKIMA SISTEMOMA V BAKTERIJI ESCHERICHIA COLI

Viktor Menart[1], Vladka Gaberc-Porekar[2], Nada Kraševec[2], Stjepan Miličić[1], Radovan Komel[2]

[1] LEK d.d., Research and Development, Celovška 135, 61107 Ljubljana, Slovenia
[2] National Institute of Chemistry, Hajdrihova 19, 61115 Ljubljana, Slovenia

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Key words: *expression plasmid; fusion protein; inclusion bodies; renaturation; chromatography*

Abstract – Background. *For biosynthesis of many recombinant proteins Escherichia coli (E.coli) is a very suitable host organism due to easy manipulation and availability of data concerning its genetics, plasmids etc. We explored the applicability of expression plasmids pMAX and pCYTEXP1 for the laboratory production of human tumor necrosis factor α (TNF).*

Methods. *For the reconstruction of plasmids and insertion of TNF gene standard methods of recombinant DNA technology were used. For isolation of the pure protein known chromatographic techniques were applied, taking into account the conditions which maintain biological activity of TNF. In assessing the quality of the purified protein, the main criteria were sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) – single band, high specific activity (cytotoxicity) and the absence of DNA and endotoxin.*

Results. *In the system E. coli JM109/pMAX-TNF, the fusion protein adenylate kinase-TNF (ADK-TNF) is accumulated within the cell as insoluble inclusion bodies. Expression level was estimated to be about 10 to 30%. Inclusion bodies were dissolved, than the ADK fragment was cleaved from TNF with CNBr. Crude mixture was separated on preparative SDS-PAGE. TNF was electroeluted from the gel and in the same time renatured in a dialysis bag. We obtained a pure protein (single band on SDS-PAGE) having specific activity of 1–2 × 10^6 U/mg. Purification yield was 30–40%. With the system E. coli TG1/pCYTEXP1-TNF, a stable expression of soluble, biologically active TNF was achieved at the level of 1–5% of total cytoplasmic proteins. For purification we applied anionic chromatography, hydrophobic interaction chromatography (HIC) and preparative non-denaturing PAGE as the main separation steps. The final yield of TNF, having high specific activity of 2–3 × 10^7 U/mg, was 10–20%. The content of endotoxin, determined by Limulus Amebocyte Lysate (LAL) test, was less than 0.1 ng/mg of protein and the content of DNA was below the detection limit.*

Conclusions. *After the insertion of TNF gene, both expression plasmids used retained their structural stability and are suitable for laboratory preparation of mg amounts of TNF. Isolation of TNF, starting with inclusion bodies of fusion protein (pMAX), is easier and relatively rapid, yet it will be necessary to improve the renaturation step so that also the requested specific activity will be achieved. In the case of direct expression of soluble TNF in the cytoplasm*

Ključne besede: *ekspresijski plazmid; fuzijski protein; inkluzijska telesa; renaturacija; kromatografija*

Izvleček – Izhodišča. *Za biosintezo manjših količin mnogih rekombinantnih proteinov je bakterija Escherichia coli zelo primerna zaradi enostavne manipulacije in večinoma dostopnih podatkov o genetiki, plazmidih itd. Preizkusili smo uporabnost ekspresijskih plazmidov pMAX in pCYTEXP1 za produkcijo humanega faktorja tumorske nekroze (TNF)-α. Razvili smo ustrezne metode za izolacijo TNF, izhajajoč v enem primeru iz inkluzijskih teles biološko neaktivnega fuzijskega proteina in v drugem primeru iz citoplazme, ki je vsebovala topen, biološko aktiven TNF.*

Metode. *Za preoblikovanje plazmidov in vključitev gena za TNF smo uporabili standardne metode tehnologije rekombinantne DNA. Pri čiščenju smo uporabili znane kromatografske tehnike z upoštevanjem pogojev, ki ohranjajo biološko aktivnost TNF. Za oceno kvalitete očiščenega proteina smo kot glavne kriterije vzeli prisotnost ene same lise na poliakrilamidni gelski elektroforezi z Na dodecil sulfatom (SDS-PAGE), visoko specifično aktivnost (citotoksičnost) ter odsotnost DNA in endotoksinov.*

Rezultati. *V ekspresijskem sistemu E. coli JM109/pMAX-TNF se fuzijski protein adenilatna kinaza-TNF (ADK)-TNF izloča v obliki netopnih inkluzijskih teles. Nivo ekspresije je ocenjen na 10–30%. Inkluzijska telesa smo izolirali in raztopili, ADK fragment pa odcepili od TNF s CNBr. Iz nastale zmesi smo izolirali TNF z uporabo SDS-PAGE. Iz gela smo TNF eluirali in hkrati renaturirali v dializni cevki. Dobili smo čisti protein (ena lisa na SDS-PAGE) s specifično aktivnostjo 1–2 ×10^6 U/mg. Izkoristek po čiščenju je bil 30–40%.*
S sistemom E. coli/pCYTEXP1-TNF smo dobili stabilno ekspresijo biološko aktivnega TNF v citoplazmi, 1–5% celotne vsebnosti citoplazemskih proteinov. Pri izolaciji smo uporabili več zaporednih korakov: anionsko kromatografijo, nativno PAGE, hidrofobno interakcijsko kromatografijo (HIC). Izkoristek čiščenja je bil 10–20%. Dobili smo čisti protein s specifično aktivnostjo 2–3 × 10^7 U/mg. Vsebnost endotoksinov je bila pod 0,1 ng/mg proteina, vsebnost DNA pa pod mejo detekcije.

Zaključki. *Oba preizkušena ekspresijska plazmida sta po vstavitvi TNF gena strukturno stabilna in uporabna za laboratorijsko pripravo miligramskih količin čistega proteina. Izolacija TNF prek fuzijskega proteina ADK-TNF (pMAX) je sicer enostavnejša, treba pa bo še izboljšati fazo renaturacije proteina, da bo dosežena tudi potrebna specifična aktivnost.*

(pCYTEXP1), a more elaborated and time consuming purification procedure is needed, anyway, the final protein is very pure and is equivalent in quality to commercially available preparations. This system is being currently used in our laboratory for the preparation of TNF analogs.

Pri ekspresiji topnega TNF neposredno v citoplazmi (pCYTEXP1) je sicer potrebna daljša in zahtevnejša pot za izolacijo, vendar je pridobljeni protein glede kvalitete enakovreden pripravkom, ki so na trgu. Ta sistem sedaj tudi uporabljamo za pripravo analogov TNF.

Introduction

In theory, any foreign gene can be expressed in *E. coli,* but expression can vary substantially, from barely detectable to very high levels. Cytoplasmic and secretory proteins, which do not have many disulfide bonds and do not require specific glycosylation pattern for biological activity or stability, can be usually well expressed in *E. coli*. With complex membrane proteins and those extremely toxic to the host cell troubles such as low expression, instability etc. could be expected and specialized expression plasmids with tightly controlled promoters are usually needed.

In general an expression level of 1–5% of total cytoplasmic proteins is relatively easy to obtain, which is sufficient to supply the material for a laboratory scale isolation of mg amounts of the protein. Problems usually arise when a very pure substance, i.e. pharmaceutical grade, is requested. In such a case the development of economic procedures is more time consuming. However, the above mentioned expression level in laboratory flask culture is also a good starting point for optimization in a small laboratory fermentor.

There are three general approaches to produce a foreign protein in *E. coli*.

1) Direct expression of soluble biologically active protein in cytoplasm or insoluble, biologically inactive protein, deposited in the form of inclusion bodies. High accumulation is possible with larger proteins (more than 100 amino acid residues), while smaller proteins (less than 50 amino acid residues) are usually proteolytically unstable. Resistance to proteases might be achieved by fusion to a larger native *E. coli* protein (for instance β-galactosidase) as discussed below. Proteolytic activity can also be partially avoided by growth at lower temperature (≈30 °C). By temperature shift it is also possible to regulate the solubility to some extent or to promote the creation of inclusion bodies.

2) Expression of the fusion protein. The protein is coupled to N- or C-terminus of another protein, which is chosen in accordance with what we want to achieve – for instance higher expression, resistance to proteases, production of inclusion bodies and so on. In many cases a small peptide is added to change the properties of the recombinant protein for its easy and specific separation from a complex protein mixture. In all these cases the fusion protein must be cleaved after being purified in order to release the target protein. For this purpose, the cleavage site (recognition sequence) for specific endoprotease must be inserted. However, cleavage itself is not always absolutely specific and does not give 100% yields. If the target protein does not contain methionine residue, the latter is inserted between two peptides to make cleavage with CNBr possible.

Some proteins are accumulated in the cytoplasm as insoluble aggregates – the so called inclusion bodies. The process is not completely understood and is still unpredictable. Higher accumulation of recombinant protein is possible because the foreign protein is currently removed and does not disturb the host cell metabolism. The formation of inclusion bodies can be useful for easier protein purification, especially in cases when final renaturation of the protein is efficient.

3) The third approach is the application of secretion expression vector. A leader peptide coupled to N-terminus of a protein to be produced enables the transfer of the foreign protein through the membranes into the periplasmic space or even into the media. If a good secretion and exact processing of the leader peptide is achieved this could be the method of choice especially for expression of smaller peptides. The purification of expression products might be simpler in such cases. However, *E. coli* is naturally a poor secretor and secretion of foreign proteins is often even less effective.

To summarize, for an unknown protein it is still not possible to predict exactly its behavior inside the *E. coli* cell and similarly it is not possible to say much in advance about its stability and expression level. So, at the beginning it is necessary to test a few different expression plasmids and hosts to estimate which one is the most promising for further optimization in the fermentor. The choice of the expression system defines to a great extent also the purification procedure which follows.

TNF-α monomer is a 17 kDa (157 amino acid residues) nonglycosylated protein, which contains a single intramolecular disulfide bond and lacks any methionine residue. Biologically active is as a compact trimer. It is soluble in *E. coli* cytoplasm and also quite resistant to proteolytic degradation. Recombinant TNF was first isolated from *E. coli* in the year 1984 (1, 2). Later on other expression systems and purification methods were developed (4, 5), including either conventional or methylotrophic yeast and *Streptomyces sp.* Production levels of few mg/L in *E. coli* to more than 1 g/L in *P. pastoris* of fermentation broth were published (3). However, we wish to stress that the price of recombinant TNF on the market has not changed over the last five years and it still remains to be about 500 DM/10 μg. So it is obvious that if larger quantity of TNF is needed, a home-made preparation could be more economic.

We tested different *E. coli* expression plasmids and two of them are presented here. pMAX is useful for the production of insoluble ADK-TNF fusion protein, and pCYTEXP1 enables the production of soluble, biologically active TNF.

Materials and methods

E. coli strains JM109 and TG1 were used for transformation and expression studies. Plasmid BBG4 bearing synthetic human TNF-gene with codons optimized for *E. coli* was supplied by British-Biotechnology. Plasmid pMAX was provided by Prof. T. Samejima (Tokyo, Japan). Plasmid pCYTEXP1 was supplied by Medac (Hamburg, Germany). Plasmid pCYTEXP1 is an improved derivative of pJLA60X series (6). Standard procedures (7, 8) were used for all manipulations of plasmids using enzymes supplied by Boehringer (Mannheim) or Pharmacia. Isolation and purification of the recombinant TNFs followed conventional chromatographic methods as described elsewhere (9) and as represented in figures. Protein concentration was estimated by Bradford procedure (10) using bovine serum albumin (BSA) as a standard. SDS-PAGE was performed according to the method of Laemmli (11) using 4% stacking gel and 15% separation gel in a vertical electrophoresis system of Pharmacia. Proteins were visualized by partially modified procedures described for silver staining and for staining with Coomassie Brilliant Blue R 350 as described in the manufacturer's instruction guide for the PhastSystem (Pharmacia).

Western blot analysis was performed according to the general procedure from the laboratory manual of Sambrook et al. (8). After SDS-PAGE the proteins were electrophoretically transferred to the nitrocellulose membrane in the NovaBlot system (Pharmacia). The nonspecific binding sites on the membrane were blocked by 3% BSA in tris buffered saline (TBS). The membrane was incubated in the solution of rabbit anti-h TNF (Sigma) at a dilution of 1:500,

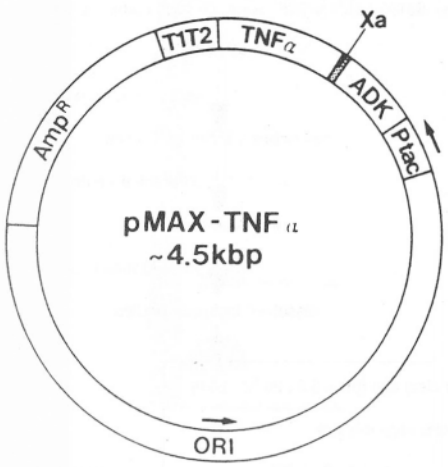

Fig. 1. *Expression plasmid pMAX-TNF.*

Sl. 1. *Ekspresijski plazmid pMAX-TNF.*

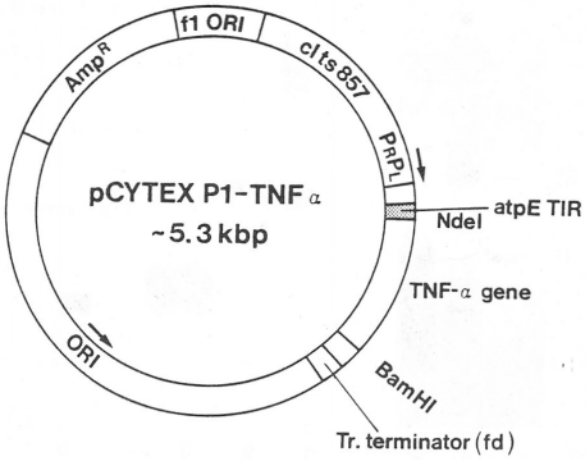

Fig. 2. *Expression plasmid pCYTEXP1-TNF.*

Sl. 2. *Ekspresijski plazmid pCYTEXP1-TNF.*

ORI: origin of replication
Amp^R: resistance to ampicillin
P_{tac}: tac promoter is usually fully induced by the addition of isopropylthio β-D-galactoside (IPTG); in our case the addition of IPTG was not necessary (leaky promoter)
TIT2: transcriptional terminator
ADK: about one half of the adenylate kinase gene (N-terminal part) which is highly expressed under the control of tac promoter
X_a: a recognition sequence (Ile Glu Gly Arg) inserted between ADK and TNF for specific cleavage by coagulation factor X_a
TNF-α: gene is inserted so that the following fusion protein results:

ADK–Met Val Ile Glu Gly Arg Met Ile Pro Met–TNF

Cleavage of the fusion protein by X_a results in TNF having 4 additional amino acid residues at N-terminus (Met Ile Pro Met-TNF).

Cleavage with CNBr produces TNF with an authentic N-terminus.

ORI: origin of replication
f1 ORI: generation of single stranded DNA after infection by M13KO7 helper phage
Amp^R: resistance to ampicillin
P_RP_L: tandem promoter from bacteriophage λ
cIts 857: thermosensitive repressor controlling the promoter; induction is achieved by temperature shift (30°C → 42°C)
Tr.terminator (fd): transcriptional terminator
atpE TIR: translational initiation region which promotes (facilitates) translation
TNF-α: gene is inserted so that the authentic protein is produced

which was followed by incubation in the solution of 1:3000 diluted goat anti-rabbit IgG coupled to horseradish peroxidase (Bio-Rad). Detection was performed by 4-chloro-1-naphtol or 3.3'-diaminobenzidine.

The bioactivity of TNF in bacterial extracts and fractions from purification procedures was measured by an in vitro cytotoxicity assay using L929 as the target cell (12).

The presence of endotoxins was controlled by Limutester Gelation Capillary Method (Funakoshi Co., Ltd., Japan) according to the supplier's instructions. The method is based on the finding of J. Levin and F. B. Bang, that endotoxins induce gel formation with Limulus Amebocyte Lysate (13). Traces of host DNA eventually present in purified TNF samples were determined by dot-blot hybridization of ^{32}P-radiolabeled random fragments from *E. coli* genomic DNA to immobilized nucleic acids on nylon membranes. Principles of the method are described elsewhere (8).

Results and Discussion

Synthetic TNF gene with codons optimized for *E. coli* was properly inserted into expression plasmids pMAX and pCYTEXP1, the basic features and properties of which are summarized in Fig. 1 and Fig. 2. For both plasmids, a selection pressure of ampicillin (50–100 μg/ml of Luria-Bertani [LB] media) was necessary to maintain appropriate plasmid copy number. Otherwise, no structural instability was observed, as concluded from plasmid preparations analyzed on agarose gels (results not shown).

In the expression system *E. coli* JM9/pMAX-TNF, a great deal of proteins appeared in the form of inclusion bodies, in which the majority (30–50% of all proteins) corresponded to ADK-TNF fusion protein (Fig. 3 a, lane 2). After treatment of dissolved inclusion bodies with CNBr, the fusion protein was completely cleaved as shown in Fig. 3 a, lane 3. The *E. coli* proteins were also cleaved, but to fragments smaller than TNF monomer. For cleavage with CNBr, much gentler reaction conditions were applied (Fig. 4) in comparison with the standard procedure (14). Pure TNF was isolated by means of semipreparative SDS-PAGE and electroelution of the appropriate protein band from the gel. After renaturation by 3-days dialysis, a final product exhibited the specific activity of $1–2 \times 10^6$ U/mg.

Attempts to cleave the fusion protein, after SDS-PAGE isolation (Fig. 4), with coagulation factor X_a were not very successful. Only 10–20% of the cleaved product was demonstrated (results not shown).

In the system *E. coli* TG1/pCYTEX1-TNF, expression of biologically active TNF in the soluble form resulted in 1–5% accumulation of the protein in cytoplasm. As seen from Fig. 5 and Fig. 6, an elaborate purification procedure consisting of several purification steps, was applied to purify and isolate TNF. We used this rather complicated and time consuming purification scheme, because we did not succeed to isolate a TNF of proper, pharmaceutical grade quality by following the already published procedures from the literature. However, regarding the prices of recombinant TNF on the market, our purification procedure resulting in a high quality TNF preparation, is still advantageous for isolation of mg amounts of this protein.

Concerning the purity of TNF preparations, TNF recovered according to both isolation procedures (Fig. 4 and Fig. 5) is essentially free from other proteins. As estimated from SDS-PAGE, at least 98% of total protein corresponds to TNF. On overloaded gels (5–10 μg TNF/well), no detectable amount of *E. coli* proteins can be spotted by visual inspection after silver staining. An immuno-reactive protein band (34–35 kDa) appeared in different amounts and corresponded probably to non-reducible TNF dimer (15). However it did not exceed 1–2% of total protein amount (Fig. 3b; lane 3).

In partially purified samples different amounts of TNF degradation product are usually found (an immuno-reactive band running in front of TNF monomer on SDS-PAGE; results not shown). The

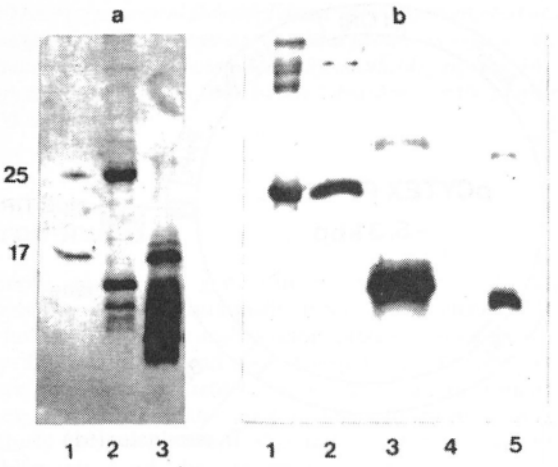

Fig. 3. *a) SDS-PAGE analysis, b) Western blot analysis.*

Sl. 3. *a) sds-PAGE analiza, b) Western blot analiza.*

1) Molecular weight standards: myoglobin (17 kDa) chymotrypsinogen (25 kDa)
2) Proteins of dissolved inclusion bodies of *E. coli* JM109/pMAX-TNF
3) Proteins of dissolved inclusion bodies after CNBr cleavage
1) Proteins of dissolved inclusion bodies of *E. coli* JM109/pMAX-TNF
2) Purified fusion protein
3) TNF-α purified from CNBr cleavage mixture
4) Molecular weight standards
5) TNF-α standard (analog Δ3N-TNF)

amount of this degradation product increases with time if samples are stored at room temperature or in inappropriate buffers. TNF degradation product can be completely removed on Phenyl Superose column (Fig. 5 and Fig. 6).

The content of endotoxin (LAL test) was less than 0.1 ng/mg of protein. Traces of host DNA were determined in 5 μg of samples and turned out to be below the detection limit (<100 pg of DNA). Concerning storage and stability of preparations, the purified protein, if stored in phosphate buffered saline (PBS) or Tris buffer (pH range from 6.5 to 8) in concentrations around 1 mg/ml is rather stable at –70 °C, which means that at least 6 months cytotoxicity is not substantially reduced. On the contrary, we have found that partially purified preparation is sometimes more susceptible to decomposition in comparison to the pure substance. Storage in non-optimal buffers or at elevated temperatures results in appearance of oligomeric species. Subunits in these aggregates are held together by covalent bonds – but not disulfide bonds (15). The formation of these aggregates can be avoided to a great extent by choosing appropriate buffer and storage conditions (15).

Conclusions

We explored the applicability of two *E. coli* expression vectors for the production of TNF on the laboratory scale.

In the case of *E. coli* JM109/pMAX-TNF, the fusion protein ADK-TNF was expressed at high levels and accumulated in the form of insoluble inclusion bodies. After solubilization in SDS-PAGE buffer effective cleavage of fusion protein with CNBr was achieved and only partial cleavage with coagulation factor X_a was demonstrated. A short and easy purification procedure was applied and the final yield of the pure TNF was about 30–40%. Unfortunately, the renaturation step turned out to be less efficient so that specific activity was not more than 1–2 × 10^6 U/mg.

In the system *E. coli* TGl/pCYTEXP1-TNF, expression of biologically active TNF directly in the soluble form resulted in 1–5% accumulation of the protein in cytoplasm. A much more time consuming procedure was needed to isolate the pure protein and final yield was only 10–20%, however, the quality of the product concerning specific activity (2–3 × 10^7 U/mg), endotoxin and DNA

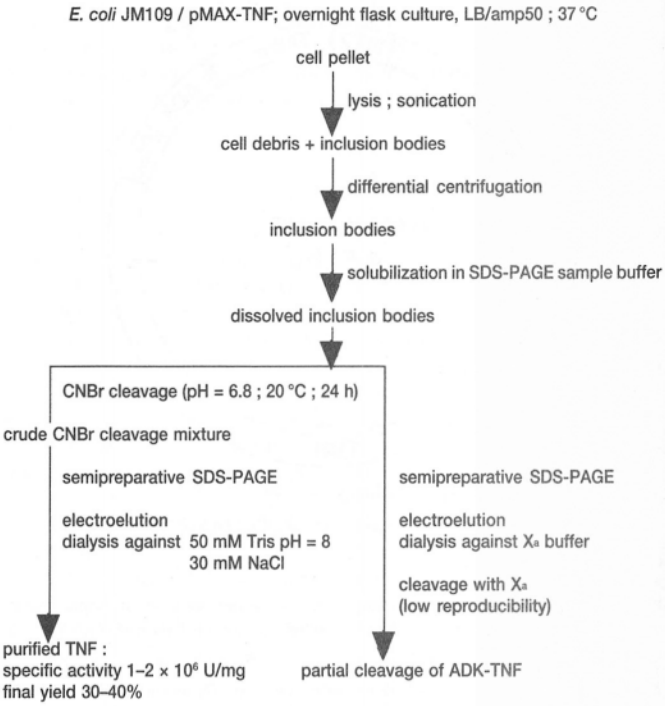

Fig. 4. *Isolation of TNF from insoluble fraction (inclusion bodies) – a simplified scheme.*

Sl. 4. *Izolacija TNF iz netopne frakcije (inkluzijskih teles) – poenostavljena shema.*

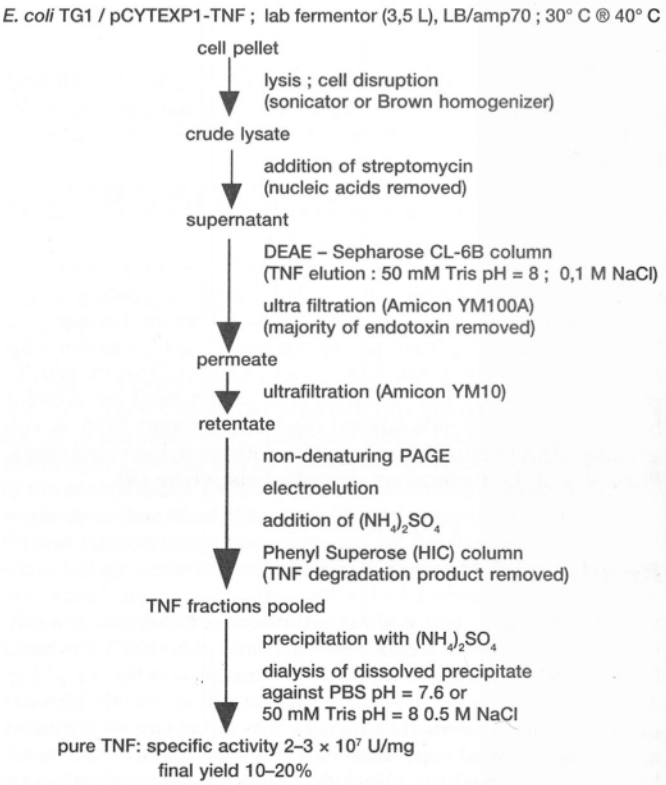

Fig. 5. *Isolation of TNF from cytoplasmic fraction – a simplified scheme.*

Sl. 5. *Izolacija TNF iz citoplazemske frakcije – poenostavljena shema.*

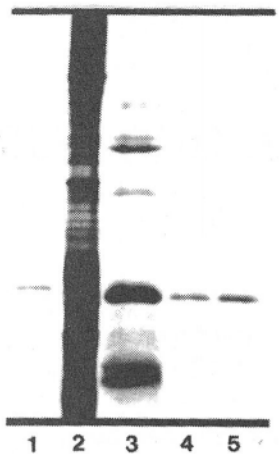

Fig. 6. *SDS-PAGE analysis at differentpurifacation steps.*

Sl. 6. *SDS-PAGE analiza po različnih stopnjah čiščenja.*

1) Molecular weight standard : myoglobin (17 kDa)
2) Crude lysate of *E. coli* TG1/pCYTEXP1-TNF
3) TNF-a rich fraction after separation on DEAE Sepharose CL-6B
4) TNF-a after semipreparative non-denaturing PAGE
5) tnf-a AFTER pHENYL-sUPEROSE (hic)

content was equal or comparable to commercial preparations. The content of endotoxin (LAL test) was less than 0.1 ng/mg of protein and the content of DNA was below the detection limit.
The latter expression system is now successfully used in our laboratory for mutagenesis and for laboratory preparation of new TNF analogs.

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References

1. Wang AM, Creasey AA, Ladner MB et al. Molecular cloning of the complementary DNA for human tumor necrosis factor. Science 1985; 228: 149–54.
2. Pennica D, Nedwin GE, Hayflick JS et al. Human tumor necrosis factor: precursor structure, expression and homology to lymphotoxin. Nature 1984; 312: 724–9.
3. Sreekrishna K, Nelles L, Potenz R et al. High-level expression, purification, and characterization of recombinant human tumor necrosis factor synthesized'in the methylotrophic yeast *Pichia Pastoris*. Biochem 1989; 28: 4117–25.
4. Aggarwal BB, Kohr WJ, Hase PE et al. Human tumor necrosis factor: production, purification and characterization. J Biol Chem 1985; 2260: 2345–54.
5. Lin LS, Yamamoto R. Purification of native and recombinant tumor necrosis factor. In Protein Purification: Micro to Macro (Ed. Burgess R), Alan R. Liss, Inc., New York, 1987: 409–19.
6. Schauder B, Blöcker H, Frank R, McCarthy JEG. Inducible expression vectors incorporating the *Escherichia coli* atpE translational initiation region. Gene 1987; 52: 279–83.
7. Ausubel FM, Brent R, Kingston RE, Seidman YG, Smith JA, Struhl K. Current protocols in molecular biology. Greene Publishing Associates and Wiley-Interscience, 1989.
8. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning 3. A laboratory manual. Cold Spring Harbor Laboratory Press, USA, 1989: 18.60–18.75.
9. Harris ELV, Angal S ed. Protein purification methods: a practical approach. IRL Press, Oxford, 1989.
10. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248–54.
11. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227: 680–5.
12. Matthews N, Neale ML in Lymphokines and Interferons (Ed. Clemens MJ, Morris AG, Gearing AJH), IRL Press, Oxford, Washington, 1987: 221–5.
13. Levin J, Bang FB. The role of endotoxin in the extra-cellular coagulation of limulus blood. Bull Johns Hopkins Hosp 1964; 115: 265–5.
14. Gross E, Witkop B. Nonenzymatic cleavage of peptide bonds: The methionine residues in bovine pancreatic ribonuclease. J Biol Chem 1962; 237: 1856–60.
15. Hora MS, Rana RK, Smith FW. Lyophilized formulations of recombinant tumor necrosis factor. Pharm Res 1992; 9: 33–6.

Professional article/Strokovni prispevek

NATURAL AND ARTIFICIAL IMMUNOMODULATION

NARAVNA IN UMETNA IMUNOMODULACIJA

Vladimir Kotnik[1], Mojca Čižek-Sajko[1], Alojz Ihan[1], Saša Simčič[1], Branka Wraber[1], Anton Štalc[2]

[1] Institute of Microbiology, Medical Faculty Ljubljana, Korytkova 2, 61105 Ljubljana, Slovenia
[2] LEK d.d., Research and Development, Celovška 135, 61000 Ljubljana, Slovenia

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Key words: *lymphocytes; Interleukin 2; soluble Interleukin 2 receptor; NK cell; chromatography*

Abstract – Background. *Immunomodulation is an natural event. It is important in the development of the disease and healing. Understanding of these natural events leads to the production of natural and artificial substances useful for the therapy. Some of them can increase, some decrease the selected immune defence activities. A lot more is known about immunosuppressants than about immunostimulators. In the presentation the authors discuss some natural and artificial immunomodulatory mechanisms to elucidate the complexity of these events, supporting their opinion with experimental data. First they discuss Il-2/soluble Il-2 receptor system, where attempt was made to present the regulatory role of soluble Il-2R molecule. Next the authors discuss the problem of target recognition by NK cells. The possible involvement of an IFN gamma-like molecule originating from the target cell is proposed to be of significance.*

Ključne besede: *limfociti; interlevkin 2; topni receptor za interlevkin 2; celice NK; kromatografija*

Izvleček – Izhodišča. *Naravna imunomodulacija je eden od bistvenih homeostatskih procesov v organizmu. Navadno teče samodejno, nanjo pa lahko vplivamo tudi od zunaj, umetno. Imunski odziv je posledica interakcij med elementi imunskega sistema. Vpletene so celice, organi in topni proizvodi v krvi in medceličnini. Ustrezni receptorji na membrani imunskih celic in primerni ligandi v medceličnini omogočajo učinkovito sporazumevanje znotraj imunskega sistema. Za prenos signala od antigen predstavitvenih celic k celicam, ki bodo ta signal prepoznale na specifičen način in ga na koncu uničile, so potrebni posebno organizirani receptorji, ki ločujejo med lastnim in tujim. Imunski odziv lahko razdelimo v tri faze: začetno, osrednjo in efektorsko. Takšna razdelitev omogoča enostavnejše razumevanje zapletenih reakcij. Odkritje molekul, ki posredujejo pri prepoznavanju, omogoča razumevanje specifičnosti reakcij in pretebtanega varnostnega mehanizma, ki onemogoča uničenje antigen predstavitvenih celic v efektorski fazi. Pri tem se odpira vprašanje smiselnosti navedene organizacije v primeru znotrajcelične okužbe z mikrobi, ki zajedajo v antigen predstavitvenih celicah.*

Metode in rezultati. *Avtorji so opisali dva eksperimentalna primera poseganja v imunski sistem. V prvem so poskusili vplivati na strnjevanje in izločanje Il-2. Iz gojišča, v katerem so gojili s PHA spodbujene limfocite, so v osemurnih intervalih odstranjevali molekule topnega Il-2R. Ta receptor naj bi uravnaval sintezo novih molekul Il-2 tako, da se prične množina Il-2, rastnem gojišču po določenem času samodejno zmanjševati. Vzrok za navedeno zmanjšanje ni znan. Avtorji so predpostavili, da se Il-2 iz gojišča veže s topnimi receptorji za Il-2. Ker vodi spodbujanje membranskih receptorjev za Il-2 k povečani sintezi in sproščanju Il-2, ima zmanjšanje množine Il-2 v okolici spodbujenih celic za posledico zmanjšanje signala za sintezo Il-2 in torej manjšo množino Il-2 v supernatantu. Odstranitev molekul topnega Il-2 receptorja bi torej morala povzročiti povečanje množine Il-2, to pa povečano spodbujanje membranskih receptorjev za Il-2, in to povečano množino na novo sintetiziranega in sproščenega Il-2. V svojih poskusih so uspeli zmanjšati množino topnih molekul Il-2R v supernatantu, vendar se je istočasno zmanjšala tudi množina Il-2.*

V drugem primeru so preskušali hipotezo o spodbujevalnem vplivu tarčnih celic (K 562) na efektorske celice (celice NK). Predpostavili so, da tarčne celice sintetizirajo membranske molekule, ki se lahko sproščajo tudi v gojišče, v katerem se celice tarče razmnožujejo. Z monoklonskimi protitelesi, uperjenimi proti Il-1, Il-2, IFN alfa in IFN gama, ki so znani spodbujevalci dejavnosti celic NK, so poskušali zavreti spodbujevalno delovanje "stimulacijskega faktorja" v supernatantu nad celicami K 562. To jim je uspelo s protitelesi, uperjenimi proti IFN gama.

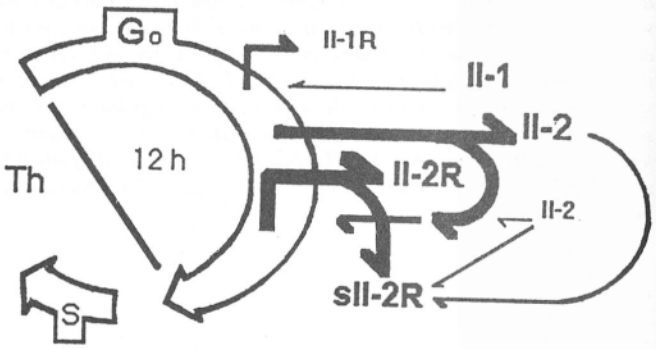
Zaključki. *V prvem primeru zaključujejo avtorji opis z mnenjem, da molekule topnega Il-2R nimajo pričakovanega uravnalnega učinka, iz do sedaj opravljenih poskusov pa še ne morejo izluščiti vzrokov, zakaj.*
V drugem primeru avtorji ugotavljajo, da celice tarče (K 562) sintetizirajo molekule, ki spodbujajo efektorske celice (celice NK) k povečani učinkovitosti. Omenjene molekule so po svojih antigenskih značilnostih podobne molekulam IFN gama.

Introduction

Enhancing or inhibitory regulation effects in the immune system are named immunomodulation. Immunomodulation is the constitutive element of the system. It is of natural origin. Without immunomodulation, the immune system will be rigid and, if exposed to harmful influences from the surrounding, very vulnerable.
Some principles of the immunomodulation are well known for a long time and of great use in prevention and healing of some serious infectious diseases like diphtheria, tetanus, small pox and others. Mechanisms of immunomodulation are still under extensive investigation. Knowledge from these studies should be used in proceedings of artificial immunomodulation.

Natural immunomodulation

The regulation patterns of the immune response are quite complicated and little understood. The statement of AM Moilin and AM Silverstein: "Regulation: The ultimate goal of immunophysiology" (1) is appropriate to stir up enthusiasm for studying immunoregulation.
The immune response can be theoretically divided in three main phases: initial, central and effector phase (2). In the initial phase, antigen couples to the surface of antigen presenting cell (APC) in a non-specific way. In the central phase, the antigen is presented to the immunocompetent cells (lymphocytes) in a specific way. Lymphocytes recognise antigens bound to APC through specially designed receptors. Receptors of this kind have two main domains important for the recognition of an antigen: first recognising major histocompatibility complex class II (MHC II) structures and the second recognising antigen specific site. This double site receptors are named T cell receptors (TCR). Presence of an TCR on the membrane of immunocompetent cell is of tremendous importance. Lymphocytes, helper cells, are activated when binding of TCR with the antigen on the APC occurs. This activation signal is, in normal circumstances, accompanied by other signals. Some of the signals are transferred by soluble molecules, lymphokines. For the activation of helper cells interleukin 1 (Il-1) is of great importance. Activated helper cells are translating information on presence of an antigen to other lymphoid cells, communicating with them with other lymphokines (3), like interleukin 2 (Il-2), interleukin 4 (Il-4), interleukin 5 (Il-5), interleukin 6 (Il-6) and interferon gamma (IFNg). About hundred different molecules are recognised today to be relevant for the perfect function of the immune system. They are expressed on the surface of the cells involved, and released from them. Not only lymphocytes but also cells from the supportive tissue, endothelial cells of vessels and some organs constitutive cells are involved. After accepting the stimulatory signal, these cells differentiate and proliferate into the mature effector cells, which finally demonstrate their activity in the effector phase of the immune reaction. The effector phase is characterised with the production of specific antibodies, toxic mediators or cytotoxic action of specialised killer cells.
The most known molecules responsible for the modulation of the immune response are comprehended in groups of cytokines (soluble products of non-lymphoid cells), monokines (soluble products of monocytes and macrophages) and lymphokines (soluble products of lymphocytes, but not antibody molecules) (4, 5). These molecules have their corresponding receptors on the immune response associated and the immune competent cells. They are of different construction. Basically, they have one more or less specific site able to bind to the specific region of the receptor molecule, and another non-specific site of importance for the constitutive effect of the effector molecule. Receptors are usually composed of the external, for the ligand specific part, part anchored in the membrane, important for the signal transmission from the specific region to the intracellular region, and intracellular part which activates intracellular activation molecules. Although the receptors are specific to the certain kind of cytokine, there are similarities between some of them. This results in cross-reactivity with several other activating molecules. The phenomenon is known as the pleiotropic activity of the communication molecules of the immune regulation system (6).



Scheme 1. *Self-amplifying mechanism of Il-2 synthesis and release, controlled by binding of own and foreign Il-2 to soluble Il-2R (modified and improved from 10).*

One of the best known couples of activating and accepting molecules is the Il-2/Il-2R (R for receptor) system (7, 8, 9). Activating molecule (Il-2) is synthesised by activated T lymphocytes. It is a single polypeptide of Mr 15,420 constituted of 133 aminoacid residues when secreted (10). The Il-2 molecule acts on its targets in a sense of an growth factor. To be effective, it should couple to the Il-2R molecule. The Il-2R molecule is expressed on activated T-lymphocytes, B-lymphocytes, thymocytes, macrophages and natural killer cells (NK cells). Resting cells do not express Il-2R. Nine hours after activation of the cell, it is expressed on the membrane (11). Its expression peaks between 48 and 72 hours (12). Il-2R is constructed of two polypeptide chains, a smaller (Mr 55,000) alpha chain and a bigger (Mr 75,000) beta chain. Beta chain of Il-2 receptor is called intermediate affinity receptor and represents 90% (30,000 to 60,000 molecules per cell) of the Il-2R molecules expressed on activated T lymphocytes. The alpha chain, or low affinity receptor, is present on the membrane only if associated with beta chain, constructing Il-2 high affinity receptor (10% or 2,000 to 4,000 molecules per cell). Beta chain binds Il-2 slowly but retains it longer than alpha chain, alpha chain binds Il-2 rapidly but releases it quickly. When both chains are in the complex, they bind Il-2 quickly and release it slowly, giving the

cell opportunity to receive the signal from Il-2 molecule. Beta chain mediates the signal, while alpha chain helps in binding the ligand (10). The alpha chain contains a specific portion recognised by anti-Tac monoclonal antibody. The alpha chain can be released from the membrane and found in the extra cellular fluids as the soluble Il-2R with, perhaps, important regulatory properties. Recently the gamma chain of the Il-2R was discovered (13, 14). This chain (Mr 64,000) facilitates binding of Il-2 to beta chain.

Artificial immunomodulation

Great effort was made in recent years to transfer the knowledge of immunomodulatory mechanisms to medical praxis (15). But its application in therapeutical approaches did not bring any great success, contrary to what was expected.

In our laboratory, experiments were performed to elucidate the role of soluble Il-2R in regulation of Il-2 release. We proposed, that soluble Il-2R form binds, though with low affinity, enough Il-2 to restrict its binding to high affinity receptors on the membrane of activated lymphocytes, if they are already saturated with Il-2. Because of the property of the alpha chain to release bound Il-2 molecules only slowly, it remains unavailable for high affinity receptors, when they are able again to bind Il-2. This way it suppress the activation of Il-2R bearing cells and the production of new Il-2 molecules. The end result is then a stop of the immune response.

We prepared a CNBr activated Sepharose gel coated with antibodies against soluble Il-2R molecules (anti-Tac). With the use of this gel, soluble Il-2R molecules could be removed from the supernatant of the activated T-lymphocytes in cell culture. After such an treatment of supernatant the soluble Il-2R should not be available any more for binding of Il-2, but the Il-2 would be available to bind to the membrane bound receptor. This way positive activation loop for the production of Il-2 would be constructed, and a constantly increasing amount of Il-2 in the supernatant expected.

The results presented in Fig. 1 give the information on the dynamics of Il-2R and Il-2 changes in our system. The concentration of Il-2 normally increases in the first 48 hours after PHA stimulation of lymphocytes in culture, and after 48 hours begins to decrease by itself. The dynamics of Il-2R increase is different from that described for Il-2. Il-2R concentration increase rapidly for 40 hours when the increase becomes slower, but it is still evident after 72 hours. After chromatography on the anti Il-2R gel, the dynamics is different. Il-2R molecules were bound to anti Tac antibodies on CNBr activated Sepharose and efficiently removed from the applied supernatant every time when the supernatant was chromatographed, which was not the case with the control supernatant, which was chromatographed over a "blind" (BSA) column. In the same supernatants, also the concentration of Il-2 was measured. Its concentration was measurable only at the beginning of the experiment, but as the chromatographic procedure continued it was almost undetectable. The results were the same whether the anti-Tac-or BSA-loaded column was used. The disappearance of Il-2 was explained in two ways: first with the proposition that Il-2 binds to the Il-2R molecules bound to gel, second that it binds also to free, with anti-Tac or BSA non-occupied spaces of CNBr Sepharose gel (12).

The results presented are indicative for the conclusion that the significance of the soluble Il-2R in regulation of Il-2 synthesis and its release is not substantial. But perhaps the findings will be of interest in some diseases where defect of the Il-2R gene exist. This defect results in 5 to 10 times bigger amount of Il-2R in the serum of the patient. If the correlation between pathogenicity and concentration of Il-2R will be found, our experiment procedure could be of value for therapeutica treatment of such disease (16).

In the experiments described next, the possibility to influence the activity of natural killer (NK) cells was studied. NK cells are able

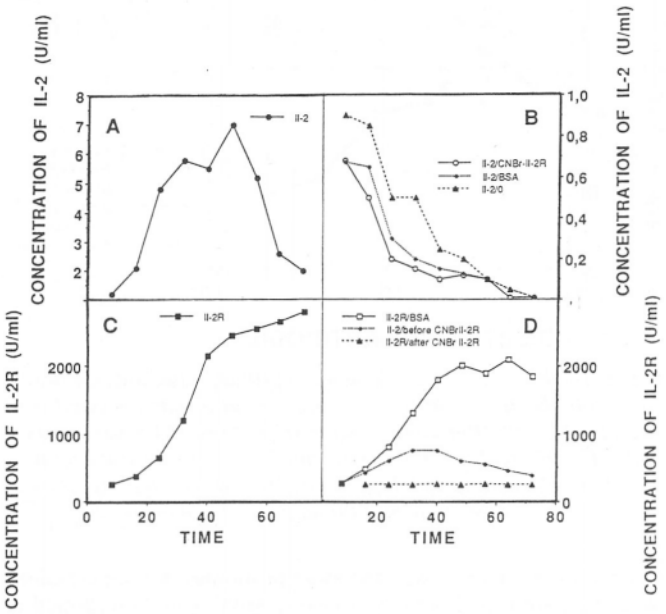


Fig. 1. *The Il-2 and Il-2R containing supernatant over continuously growing PHA-stimulated primary human lymphocyte culture was applied every 8 hours to anti-Il-2R or BSA-coated CNBr activated Sepharose gel. Chromatographed supernatant was then returned to the original cell culture flask and growing of lymphocytes continued for another 8 hours. The process was repeated every 8 hours for 72 hours. Panel A: Time-dependent changes of Il-2 concentrations in supernatant of a normal PHA-stimulated lymphocyte culture. Panel B: Time-dependent changes of Il-2 concentrations in the same supernatant after chromatography on anti-Tac or BSA-coated CNBr Sepharose. Panel C: Time-dependent changes of Il-2R concentrations in supernatant of a normal PHA stimulated lymphocyte culture. Panel D: Time-dependent changes of Il-2R concentrations in the same supernatant after chromatography on anti-Tac or BSA-coated CNBr Sepharose.*

to kill tumour cells without prior sensibilization. Tumour cells differ in their susceptibility for NK cells attack, K 562 cell line being the most susceptible in the human system. How NK cells are triggered to kill tumour cells is still under investigation (17, 18, 19). Different possibilities were discussed in the past. One of them proposes the presence of a specific receptor. The receptor was actually found several years ago and named T9 receptor (2, 20). Until now, this receptor was not isolated or characterised. Based on this proposition, it can be shown experimentally, that priming of freshly isolated NK cells with K 562 cells stimulates the former to be more effective in killing fresh K 562 targets (17). We tried to find out whether the supernatant over the K 562 cells growing in the culture had the same priming activity. If that were the case, the "stimulating" receptor molecule in a soluble form could be postulated, and perhaps isolated from the supernatant, and then used as an NK cells activating factor.

We grew K 562 cells in optimal culturing conditions until they began to deteriorate. This happened normally on day 5 to 7. The supernatant was then separated from the cells with decantation and centrifugation at 500 g for 10 minutes. Supernatants were frozen at –20°C and used after thawing in subsequently described experiments.

Addition of already mentioned supernatant to freshly isolated human lymphocytes for 20 hours resulted in a dose-dependent increase of NK cells mediated killing activity of K 562 targets. This stimulatory activity was present in most of the tested supernatants

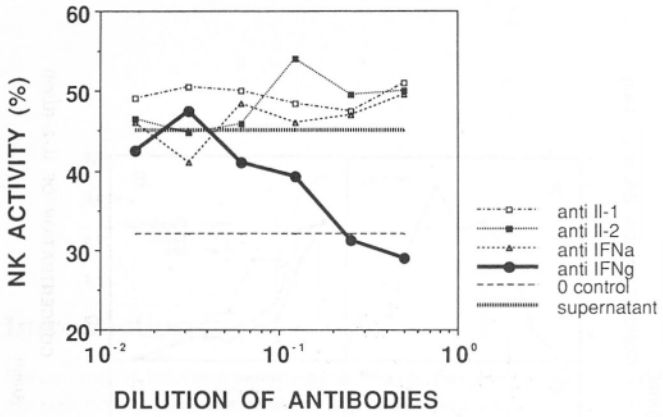


Fig. 2. *The K 562 cells were grown in optimal culturing conditions until they began to deteriorate. Supernatant was collected and used for NK cell stimulation. Different portions of the same supernatant were treated with monoclonal antibodies raised against Il-1, Il-2, IFN alpha and IFN gamma. The NK cells activity test with 51 Cr labelled K 562 target cells was performed.*

but not in all. Activity was the most pronounced if supernatants were prepared as described above; if they were centrifuged at 10.000 g for 30 minutes, the activity was decreased or lost. This results are indicative for the opinion that the "stimulating" factor is a part of the cell membrane. To further characterize our "stimulator" factor, we compare its activity to already established NK cell stimulating molecules like Il-1, Il-2, IFN alpha and IFN gamma. The testing was performed using a thymocyte test. Recombinant human Il-2 was used as a positive control. It was found that investigated supernatant has an Il-2 like activity. To point out the factor responsible for the stimulating activity, we performed blocking experiments with monoclonal antibodies raised against Il-1, Il-2, IFN alpha and IFN gamma. All of the antibodies used were able to block the activity of the supernatant in the thymocyte test. To obtain a more relevant information, the blocking experiments were performed using also an NK test. In that case, the dose-dependent inhibition of stimulation was observed with anti-IFN gamma antibodies (21), while the other antibodies were without effect (Fig. 2).

Though the experiments performed are not completed yet, we have reasons to believe that K 562 cells posses a membrane-bound molecule involved in the recognition and stimulation processes of NK cells. Our data are an additional argument for the opinion that an active interchange reaction takes place between effector and target cells, which results in stimulation of effector cells and, afterwards, deterioration of target cells.

Conclusion

The immune response is highly sophisticated and well-regulated physiological process. The regulatory mechanisms are not totally elucidated yet, but with isolation and characterisation of signal molecules, especially lymphokines, there is more and more understanding about them. Although the investigators are still completing the data, attempts are made to use the known data in constructing effective, therapeutically usable protocols. Manipulation of naturally occurring regulatory events on the experimental level is shown and discussed above in order to give more information on possibilities to influence the immune response with immunomodulation in disease.

Acknowledgement

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References

1. Moilin AM, Silverstein AM. History of immunophysiology. In: Oppenheim JJ, Shevach E eds. Immunophysiology. New York, Oxford: Oxford University Press, 1990: 3–13.
2. Vozelj M. Temelji imunologije. Ljubljana: Državna založba Slovenije, 1985: 1–469.
3. Klampfer L, Kotnik V. Interlevkini in imunski sistem. Zdrav Vestn 1992; 61: 35–40.
4. Wraber B. Povezanost nastajanja topnih dejavnikov v makrofagih in limfocitih T s spremenjenim delovanjem imunskega sistema. Medicinska fakulteta, disertacija, Ljubljana 1992.
5. Ibelgaufts H. Lexicon Zytokine. München: Medicon Verlag, 1992: 19–259.
6. Ellingsworth LR. Effect of growth factors on immunity and inflammation. In: Oppenheim JJ, Shevach E eds. Immunophysiology. New York, Oxford: Oxford University Press, 1990: 320–36.
7. Chopra RK, Powers DC, Kendig NE, Adler WH, Nagel JE. Soluble interleukin 2 receptors released from mitogen stimulated human peripheral blood lymphocytes bind interleukin 2 and inhibit interleukin 2 dependent cell proliferation. Immunol Invest 1989; 18: 961–73.
8. Kuziel WA, Greene WC. Interleukin 2 and the interleukin 2 receptor: new insights into structure and function. J Invest Dermatol 1990; 94: Suppl 6: 27–32.
9. Leonard WJ Sharon M, Gnarra JR. Structure and function of interleukin 2 receptors. Prog Clin Biol Res 1990; 352: 179–87.
10. Klein J. Immunology. Boston, Oxford, London, Edinburgh, Melbourne: Blackwell Scientific Publications, 1990: 227–45.
11. Reske-Kunz AB, von Steldern D, Dude E, Diamantenstein T. Regulation of interleukin 2 receptor expression by interleukin 2. Scand J Immunol 1986; 23: 693–701.
12. Kosmina B, Lavrenčič A. Time-dependent correlation between binding interleukin 2 and interleukin 2 receptors. Med Razgl 1992; 31: 151–63.
13. Takeshita T, Asao H, Ohtani K, Ishii N, Kumaki S, Tanaka S et al. Cloning of the gamma chain of the Il-2 receptor. Science 1992; 257: 379–82.
14. Asao H, Kumaki S, Takeshita T, Nakamura M, Sugamura K. Il-2 dependent in vivo and in vitro tyrosine phosphorilation of Il-2 receptor gamma chain. FEBS Lett 1992; 304: 141–5.
15. Kotnik V, Štalc A. Preskušanje novega potencialnega imunomodulatorja. Zdrav Vestn 1991; 60: 477–81.
16. Waldmann TA. The Il-2/Il-2 receptor system: a target for rational immune intervention. Immunology Today 1993; 14: 264–9.
17. Farram E, Targan SR. Identification of human natural killer soluble cytotoxic factors (NKCF) derived from NK enriched lymphocyte populations: specificity of generation and killing. J Immunol 1983; 130: 1252–6.
18. Pohajdak B, Gomez JL, Wilkins JA, Greenberg AH. Tumor-activated NK cells trigger monocyte oxidative metabolism. J Immunol 1984; 133: 2430–6.
19. Phillips JH, Lanier LL. A model for the differentiation of human natural killer cells. Studies on the in vitro activation of Leu-11+granular lymphocytes with a natural killer-sensitive tumor cell, K 562. J Exp Med 1985; 161: 1464–82.
20. Kotnik V. Vpliv topnih produktov aktiviranih limfocitov na naravne celice ubijalke: spodbujanje naravnih celic ubijalk in vitro. Zdrav Vestn 1987; 56: 409–13.
21. Sia DJ. Differential regulation of interleukin 2 (Il-2) receptor expression on antigen-specific human T cell clones by Il-2. Immunol Lett 1988; 17: 13–9.

Professional article/Strokovni prispevek

CELL SURFACE MOLECULES THAT REGULATE ABILITY OF NK CELLS TO KILL THEIR TARGETS

MOLEKULE CELIČNIH POVRŠIN, KI URAVNAVAJO UBIJALSKO ZMOŽNOST CELIC NARAVNIH UBIJALK

Alojz Ihan

Institute of Microbiology, Medical Faculty Ljubljana, Zaloška 7, 61000 Ljubljana, Slovenia

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Key words: *NK cells; tumour cells; killing mechanisms; alloreactivity*

Abstract – Background. *Natural killer cells comprise a lymphocyte subpopulation able to kill tumour or virus-infected cells spontaneously, without prior sensitisation. NK cells kill their targets in a way that differs substantially from that of T lymphocytes. While cytotoxic T lymphocytes recognise foreign molecules – antigens on target cells, NK cells on contrary kill their targets, if certain self molecules are absent from the cell surface of the target cell. HLA molecules serve as "self" molecular signals, that are checked by NK cells. If NK cells fin targets that do not express an appropriate set of HLA molecules, the killing mechanism of NK cells is activated.*

Conclusions. *Very few data are available about a clinical significance of that mechanisms, but the role in post-transplantation immune events might be to reconsider.*

Ključne besede: *celice NK; tumorske celice; mehanizmi ubijanja; aloreaktivnost*

Izvleček – Izhodišča. *Celice naravne ubijalke (celice NK) so podvrsta limfocitov, ki so zmožni ubijati tumorske ali z virusi okužene celice brez predhodne imunizacije. Ugotovili so, da celice NK izbirajo svoje "žrtve" na bistveno drugačen način kot limfociti T. Medtem ko limfociti T na okuženih in tumorskih celicah prepoznavajo organizmu tuje molekule – antigene, celice NK na okuženih in tumorskih celicah preverjajo navzočnost nekaterih organizmu lastnih identifikacijskih molekul. Odsotnost takih molekul spodbudi ubijalsko aktivnost celic NK. Poskusi kažejo, da pri človeku molekule HLA prvega razreda služijo kot takšne identifikacijske molekule. Celice NK preverjajo navzočnost molekul HLA na celičnih površinah. Če naletijo na celice, ki ne izražajo ustreznih molekul HLA, take celice ubijejo.*

Zaključki. *Klinični pomen teh mehanizmov še ni jasen, gotovo pa bo potrebno preveriti njihovo vlogo pri imunskih pojavih, ki spremljajo presaditev organov.*

NK cells

In 1975 Herberman and co-workers described a subpopulation of white blood cells able to kill tumour cells "in vitro". The observation was unexpected because the killing happened without prior immunisation with the tumour cells. The capability of white blood cells to spontaneously kill tumour cells was termed "natural cytotoxicity" (1), and cells capable of natural cytotoxicity were defined as natural killer cells (NK cells).

In a subsequent studies Timonen (2) found out that the phenomena of natural cytotoxicity was mediated by a morphologically distinct lymphocyte subpopulation. Cells were distinguished from typical lymphocytes by a higher amount of cytoplasm, that contained azurophilic granules. Because of a distinct morphology cells were named "large granular lymphocytes" (LGL cells).

Several functions of NK cells were determined "in vitro": killing of tumour and virus-transformed cells, lymphokine synthesis, the regulation of heamatopoesis and the rejection of an allogenic transplant (4). However NK cell function in vivo is poorly defined.

Natural cytotoxicity was first described in humans and mice. Later it was described in many other vertebrates as well as in lower species, e.g. worms. Thus, it can be concluded that natural cytotoxicity is evolutionary very old.

The origin and development of NK cells

NK cells are formed from precursor cells in bone marrow. After bone marrow allogenic transplantation it is possible to detect NK cells of donor origin in the blood (5). Cells with the NK activity were detected in "in vitro" bone marrow cultures (6). Upon maturation in bone marrow NK cells are released in the blood. Their life span is not determined. "In vitro" labelled and injected NK cells persisted in the circulation from few days to several months (3).

Cytotoxic activity of NK cells

Cytotoxic activity of NK cells is measured by incubating NK cells with tumour target cells, labelled with a radioactive chromium. After 3–4 h incubation period, the radioactivity released from damaged tumour cells is measured (1). The released radioactivity is proportional with a NK ability to kill tumour cells.

NK killing can be distinguished into three phases. First the conjugate between NK cell and tumour cells is formed. Surface molecules on the NK cells (LFA-1 – CD 11a/CD 18 and CD2) and ligands (ICAM-1 [CD 54] and LFA-3 [CD 58] on the target cells participate in the conjugate formation. The conjugate formation is a necessary but not sufficient step of NK killing. In the second step

NK cells must specifically recognize tumour from non-tumour cells. After recognition of tumour cells the non-specific lytic mechanism is initiated. In the third lytic phase, NK cells release the content of cytotoxic granules in target cells. Cytotoxic granules contain proteases and cell toxins. The action of perforin is well defined. By polimerasing in target cell membranes perforin forms transmembrane channels. That cause osmotic lysis of target cells. In contrast, some other cytotoxic proteins cause apoptotic death of target cells (3).

The most intriguing question to be answered is, how NK cells distinguish tumour from non-tumour cells. Numerous membrane proteins that influence NK lysis of tumour cells were reported. Most of them were found to act as adhesion molecules, enabling the formation of NK cell - target cell conjugate. However, so far no protein was described, that would mediate the specific recognition of tumour cells (7).

NK killing and histocompatibility molecules

An important step in understanding the mechanism of NK killing was an observation that some tumour cells have a decreased expression of MHC molecules compared to non-tumour cells of the same origin. Furthermore it was shown that decreased MHC molecules expression make tumour cells more susceptible to NK lysis. For example, interferon by enhancing the expression of MHC molecules in tumour cells makes them more resistant to NK lysis (8). Thus it can be concluded that NK cells do not recognise foreign molecules (antigens) on target cells. Instead they recognise distinct "self" molecules, that serve as identification molecules of individuals normal cells. The presence of appropriate self identification molecules inhibit the NK killing ability. However if target cells do not express appropriate self molecules, NK cells recognise and kill their targets (9).

NK cells are able to kill not only tumour cells but also non-tumour cells of other individuals (allogenic killing) (10). Allogenic killing can be explained by the variability of self molecules among individuals. According to this hypothesis each individual has a unique variant of identification molecules, recognised by self NK cells. Allogenic killing is not possible between individuals expressing the same identification molecules. The opposite would happen if identification molecules in two individuals are not the same. Extensive genetic studies revealed, that identification molecules, recognised by NK cells, are located on the sixth chromosome in the area of histocompatibility genes (11, 12).

In our work we studied the role of HLA molecules in NK killing. For that purpose B cell lines with defective HLA molecules expression were prepared. We suggested that cells, resistant to NK cell lysis, would become susceptible to lysis, if they lost the identification molecules expression.

In experiments we used B cell lines with a defined HLA haplotypes. Mutants with defective HLA expression were prepared by irradiating cells with gamma rays and selected with distinct anti-HLA antibodies. We observed that cells defective in HLA Cw3 expression lost the resistance to NK lysis and concluded that HLA Cw3 molecules served as identification molecules, that inhibit NK cells killing ability. Mutated target cells, that lost the ability to express HLA Cw3 molecules, became susceptible to lysis by NK cells as well (13).

To confirm our observation we used target cells, susceptible to NK lysis. We tested if transfer of HLA Cw3 gene make cells resistant to NK lysis. We used mouse tumour target cells P815 as a model. Those cells are normally susceptible to lysis by human NK cells. As we suggested, the transfected cells became resistant to NK cell lysis (13).

The mechanisms of NK killing regulation by HLA molecules

The experiments indicate that HLA molecules, besides their role in antigen presentation to T lymphocytes, also play a role as identification molecules for NK cells. HLA molecules are expressed on all nucleated human cells. Their presence on cell surfaces indicate a cells resistant to NK cells, while the absence of HLA molecules indicates that target cells are changed. This is a signal for NK cells to kill such targets. The mechanism is extremely important in viral infections. Viruses often inhibit HLA molecule synthesis in infected cells. Because only HLA molecules are able to present viral antigens to T cells, cells with suppressed HLA synthesis become invisible for T cells. However, such cells are recognised as foreign by NK cells (9, 10). Tumour cells have a decreased expression of HLA molecules in order to escape the immune control mechanisms. NK cells may be appropriate protective mechanism.

There are still many questions about NK cells recognition mechanisms. During maturation in bone marrow NK cells must be instructed to recognise HLA molecules of an individual as self identification molecules. The mechanisms of such instructive phase are completely unknown yet. The selection process, similar to tymic T cell selection, might take a place. However, no experimental data are available up to now.

How NK cells recognise HLA molecules on target cells? NK cells do not have antigen receptors and some other receptor molecules must react with HLA molecules on target cells. It is not known, how such receptors distinguish distinct variants of HLA molecules. This question has also a practical implication: Can HLA tissue typing prevent the NK cell reaction against the grafted tissue? How important can be anti-graft NK cell reaction? In mice model it was shown, that NK cells mediate a rejection of a bone marrow transplant. The phenomenon is called a hybrid resistance. In hybrid resistance the heterozigote descendant for histocompatibility genes (A × B) F1 reject the bone marrow transplants of both homozygote parents (parent A and parent B) (4). The rejection is not due to the classic mechanisms of graft rejection. Because the heterozigote descendant express both sets of histocompatibility molecules, tissue of both parents is tolerated by T lymphocytes. It was shown that the hybrid resistance depends on the presence of NK cells. By use of monoclonal antibodies it is possible to deplete NK cells in the mice. The depletion of NK cells prevents the hybrid resistance. It is likely that NK cells of heterozygous descendant are instructed for the recognition of both fathers and mothers histocompatibility molecules. Bone marrow cells of each parent contain only one set of histocompatibility molecules and lack the other. Since no parental cells express all identification molecules used by descendants NK cells, the killing mechanisms are activated.

Conclusions

NK cells distinguish between self and non-self in a way, different from T lymphocytes. T lymphocytes kill if they recognise foreign molecules on target cells. On the other hand, NK cells attack if they do not recognise distinct normal, self molecules on target cells. Experiments demonstrate that HLA molecules serve as identification molecules for NK cells. Very few data are available about a clinical implications of that finding. However it will be important to elucidate the role of NK cells in transplantation events.

References

1. Herberman RB, Nunn ME, Lavrin DH. Natural cytotoxic activity of mouse lymphoid cells against syngenic and allogenic tumors. Distribution of reactivity and specificity. Int J Cancer 1975; 16: 216–29.

2. Reynolds CW, Timonen T, Herberman RB. NK cell activity in the rat. Isolation and characterization of the effector cells. J Immunol 1981; 127: 282–7.
3. Trinchieri G. Biology of natural killer cells. Adv Immunol 1989; 47: 187–376.
4. Yu YLL, Kumar V, Bennett M. Murine natural killer cells and marrow graft rejection. Ann Rew Immunol 1992; 10: 189–213.
5. Ault KA, Antin JH, Ginsburg D, Orkin SH, Rappeport JM, Smith BR. Phenotype of recovering lymphoid cell population after marrov transplantation. J Exp Med 1985; 161: 1483–502.
6. Keever CA, Pekle K, Gazzola MV, Collins NH, Bourhis JH, Gillio A. Natural killer and lymphokine activated killer cell activities from human marrow precursors. J Immunol 1989; 143: 3241–9.
7. Moretta L, Ciccone E, Moretta A, Hoglund P, Ohlen C, Karre K. Allorecognition by NK cells: nonself or no self? Immunology Today 1992; 13: 300–6.
8. Janeway CA. To thine own self be true. Current Biology 1991; 1: 239–43.
9. Raulet DH. A sense of something missing. Nature 1992; 358: 21–2.
10. Moretta A, Bottino C, Pende D, Tripodi G, Tambussi G, Viale O, Orengo A, Barbaresi M, Merli A, Ciccone E et al. Identification of four subsets of human CD3-CD16+natural killer (NK) cells by the expression of clonally distributed functional surface molecules: correlation between subset assignment of NK clones and ability to mediate specific alloantigen recognition. J Exp Med 1990; 172: 1589–98.
11. Ciccone E, Pende D, Viale O, Di Donato C, Tripodi G, Orengo AM, Guardiola J, Moretta A, Moretta L. Evidence of a natural killer (NK) cell repertoire for (allo)antigen recognition: definition of five distinct NK-determined allospecificities in humans. J Exp Med 1992; 175: 709–18.
12. Ciccone E, Pende D, Viale O, Tambussi G, Ferrini S, Biassoni R, Longo A, Guardiola J, Moretta A, Moretta L. Specific recognition of human CD3-CD16+natural killer cells requires the expression of an autosomic recessive gene on target cells. J Exp Med 1990; 172: 47–52.
13. Ciccone E, Pende D, Viale O, Ihan A, Di Donato C, Orengo AM, Biassoni R, Verdiani S, Amoroso A, Moretta A et al. Involvement of HLA class I alleles in natural killer (NK) cell-specific functions: expression of HLA-Cw3 confers selective protection from lysis by alloreactive NK clones displaying a defined specificity (specificity 2). J Exp Med 1992; 176: 963–71.

Professional article/Strokovni prispevek

MODULATION OF CYTOKINE SYNTHESIS IN HUMAN MONONUCLEAR CELL CULTURES OF DIFFERENT ORIGIN

MODULACIJA SINTEZE CITOKINOV V KULTURAH HUMANIH MONONUKLEARNIH CELIC RAZLIČNEGA IZVORA

Branka Wraber

Institute of Microbiology, Medical Faculty, 61000 Ljubljana, Zaloška 4, Slovenia

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Key words: *interleukin-1α; interleukin-1β; interleukin-2; T lymphocytes; immunosuppression; Graves' disease*

Abstract – Background. *Cytokines are small, secreted protein mediators that act as intercellular communicators between various cells of the immune system as well as between these and other cells of the organism. They are mostly synthesized and secreted by the activated cells of the immune system. Cytokine synthesis is regulated by differentiation of cells into various cytokine secreting phenotypes, and by selective activation of different types of cells to produce their characteristic cytokines. From the point of view that they are natural immune response modifiers it is very important to know how the regulation of their synthesis and secretion occurs. Especially of interest are the differences between normal and by disease or therapy altered immune system. Due to the complexity of the cytokine network, it is difficult to follow and influence cytokine production in vivo, therefore, in vitro approach to this problem is much more frequently presented.*

Methods. *The author studied modifications of the production of interleukin-1 α (Il-1α), interleukin-1 β (Il-1 β) and interleukin (Il-2) in human peripheral blood mononuclear cell (MNC) cultures. MNC were isolated from the blood of different groups of donors: healthy volunteers, patients with Graves' disease (autoimmune hyperthyreosis – AH) prior to treatment and patients after kidney transplantation receiving immunosuppressive therapy (IST) with Cyclosporin A (CsA) and corticosteroids. MNC in the cultures were activated with different amounts of mitogenic lectins, concanavalin A (ConA) and phytohemagglutinin (PHA), alone and in combination with phorbol mirystate acetate (PMA). Four modes of MNC stimulation were used in the experiments: ConA, PHA, ConA&PMA with PHA&PMA. At the end of the incubation the concentration of cytokines in cell culture supernatants was estimated by radioimmunoassay (RIA).*

Results. *Various responses in cytokine production to different activation modes were found among the three groups of blood donors. Not only quantitative differences but also different cytokine production patterns related to the cell origin were detected. Significant differences in the quantity of the production of Il-1 α and Il-2 were found in both groups of patients when compared with the production in the healthy group.*

Conclusions. *The combination of PHA and PMA was the most appropriate stimulation mode in the group of healthy donors. But is was not possible to overcome the effect of in vivo immunosuppression of IST patients MNC stimulated in vitro with PHA and PMA. In the case of AH, an appropriate stimulation is very important for demonstrating the ability of MNC to produce excessive amounts of cytokines, especially Il-2, which probably reflects the involment of this cytokine in the processes of disease progression.*

Ključne besede: *interlevkin-1α; interlevkin-1β; interlevkin-2; limfociti T; imunosupresija; avtoimunska hipertiroza*

Izvleček – Izhodišča. *Citokini so topni dejavniki beljakovinske narave manjše molekulske mase, ki sodelujejo pri komuniciranju med različnimi celicami imunskega sistema ter med le-temi in celicami drugih organskih sistemov v organizmu. Pretežno jih sintetizirajo in izločajo aktivirane celice imunskega sistema. Uravnavanje njihove sinteze poteka na eni strani z diferenciacijo celic v fenotipe, ki izločajo svoje značilne citokine, na drugi strani pa s selektivno aktivacijo celic, ki so zmožne izločati določene citokine. S stališča, da so citokini naravni modulatorji imunskega odziva, je zelo pomembno vedeti, kako poteka uravnavanje njihove sinteze in izločanja. Posebno zanimive so razlike v uravnavanju med imunskim sistemom zdravega organizma in onega s spremenjenim delovanjem imunskega sistema – bodisi zaradi bolezni ali zaradi imunomodulatorne terapije. Zaradi kompleksnosti v delovanju in medsebojnem učinkovanju citokinov je proučevanje teh mehanizmov in vivo zapleteno, zato je in vitro pristop na humanem materialu pogostejši.*

Metode. *Avtorica je proučevala modifikacije v izdelovanju interlevkina-1 α (Il-1 α), interlevkina-1 β (Il-1 β) in interlevkina-2 (Il-2) v kulturah humanih mononuklearnih celic (MNC). Celice je izolirala iz venske krvi različnih darovalcev: zdravi darovalci, bolniki po presaditvi ledvice, ki so bili na imunosupresivnem zdravljenju s ciklosporinom A (CsA) in kortikosteroidi ter bolniki z avtoimunsko hipertirozo (AH) pred zdravljenjem. Za spodbujanje celic je avtorica uporabila različne koncentracije mitogenih lektinov konkanavalina A (ConA) in fitohemaglutinina (PHA) samih ali v kombinaciji s forbolmiristatacetatom (PMA) in izbrala štiri različne načine spodbujanja: s ConA, s PHA, s ConA&PMA in s PHA&PMA. Po koncu inkubacije je s centrifugiranjem ločila celice od supernatantov, v katerih je z radioimunskimi metodami (RIA) določila koncentracijo citokinov.*

Rezultati. *Rezultati so pokazali razlike v odzivu MNC na izbrane načine spodbujanja in vitro med posameznimi skupinami. Razlike v izdelovanju citokinov so kvantitativne, obenem pa je opaziti tudi različne vzorce odzivanja na spodbujanje glede na izvor MNC. Signifikantne razlike se pojavljajo v MNC kulturah bolnikov pri izdelovanju Il-1α in Il-2, če ju primerjamo z izdelovanjem obeh citokinov v MNC kulturah zdravih.*

Zaključki. *Kombinacija PHA in PMA se je izkazala za najprimernejši način spodbujanja v opisanih eksperimentih. Vendar ta način spodbujanja MNC bolnikov z IST ni obšel učinka imunosupresivne terapije in vivo. V primeru bolnikov z AH pa je primerno spodbujanje zelo pomembno, ker odkrije sposobnost MNC za izdelovanje večjih količin citokinov, posebno Il-2, kot pa MNC zdravih darovalcev. To kaže na morebitno vpletenost Il-2 v napredovanje bolezni.*

The cytokines are important participants in immune events. Tim Mosmann defines them as a group of small, secreted protein mediators that act as intercellular communicators between various cells of immune system. From his point of view cytokines act mostly in immune compartment of the organism (1). On the other hand, Carl Nathan, another important investigator in this field, believes that the cytokine science has been progressing since the discovery of interferon in the fifties more or less separately in four different fields: immunology with interleukins, virology with interferons, hematology with colony-stimulating factors and cell biology with growth factors. Therefore, his definition of cytokines is more general and more suitable for the researchers of all four fields mentioned. He defines a cytokine as a soluble glycoprotein, nonimmunoglobuline in nature, released by a living cell of the host, which acts nonenzymatically in picomolar to nanomolar concentrations to regulate host cell function. Cytokines make up the fourth major class of soluble intercellular signalling molecules alongside neurotransmitters, endocrine hormones, and autacoids. The physiologic importance of cytokines is not lesser than that of other classes (2).

Cytokines are biological response modifiers acting as signal transducers between different cells of the organism, but to a great extent they may act between the cells of the immune system or between immune system and other organ systems in the organism. Studies with natural and recombinant cytokines revealed that most cytokines are pleiotropic and have multiple biologic activities with many cell targets which express their specific receptors (3). There is also considerable overlap of function between cytokines, and one of the best examples for this is the proliferation of T-lymphocytes which can be enhanced by Il-2, Il-4, Il-6, Il-7 and Il-9. The cytokine network is very complicated and not at all explained in details. In Mosmann's opinion, the extraordinary complexity of the cytokine network is a defence against pathogen interference. Since microorganisms can evolve more rapidly than their mammalian hosts, it would be very difficult for any single immune mechanism to perform a given function without possibility of microorganism interference. The strategy of multiple interlocking regulatory mechanisms offers extra assurance against this interference. It should be difficult for a pathogen microorganism to evolve simultaneously several different agents that were able to interfere with all the necessary components of a complex interlocked system. Thus the complexity of the cytokine regulatory network may be a defence against pathogen interference (1).

And which substances belong to the cytokines? In immunology, cytokine research was started in late sixties with the discovery of some soluble factors which were designated as lymphokines because it was thought that they were products of lymphoid cells functioning as signal transducers between these cells. Up to date, this term describes the cytokines produced by T-lymphocytes, merely T-helper, and they have a pivotal role in immune response regulation (4). The cytokines such as interleukin-1 (Il-1), tumor necrosis factor α (TNF α) and other products of macrophages are termed monokines. Soluble factors derived merely from the cells of nonleukocyte type, but displaying some effects on immune cells, are classified as cytokines (5).

Interleukin is another term in immunology which was introduced at one of the lymphokine conferences in 1979 in connection with Il-1 and Il-2 to prevent confusion in nomenclature. This terminology has been preserved until now (6). In 1991, to prevent the overflow of new interleukins, the WHO subcommittee on interleukin designation published the criteria for such designation:

1. The molecule has to be purified, cloned and expressed. Its nucleotide and amino acid sequence should be different from any other molecule already described. Neutralising antibodies and known chromosomal location would be desirable.
2. The molecule has to be natural product of cells of the immune system, be known to mediate a potentially important function in immune responses, preferably more than only one simple function, and moreover, the majority of the action should be in the immune system compartment, which is in this case defined very broadly to include leukocytes other than lymphocytes and monocytes as important participants.
3. If the molecule is a member of an already characterized family of molecules that have their major functions outside the immune system, it would be preferable to use the designation connected with that family.
4. The Subcommittee recommends the use of a well-chosen descriptive name whenever possible. A good example is TNF α (7).

There have been 13 interleukins described (8) until today, but nevertheless, Il-1 and Il-2, even though discovered more than ten years ago, are still interesting molecules recently studied by many immunologists. Il-1 is a cytokine responsible for mediating a variety of processes in host defence, inflammation and response to injury. Il-1 may be grouped with TNF α, lymphotoxin and Il-6 as factors mediating common effects. Although initially described as a product of activated macrophages (11), Il-1 is now known to be produced by practically all nucleated cells in the organism. It is a cytokine with pleiotropic properties and has various biological activities. Il-1 consists of two distinct but related molecules (Il-1 α and Il-1 β), encoded by separate genes but sharing the same receptors, and as a consequence having similar biological properties. The meaning of two distinct molecules has not yet been clarified. In the past it was thought that Il-1 is a major costimulatory signal delivered by activated macrophages and other antigen-presenting cells to activate lymphocytes and to push them into the proliferation cycle. The up-to-date opinion is that other, membrane-bound molecules play a pivotal role in these events and Il-1 stimulates the process though it is not essential (3, 12, 13).

Il-2 is secreted mainly by T-helper cells; the process begins 4–12 hours following activation by binding of an antigen to the T-cell receptor and appropriate costimulation. Approximately at the same time, the high-affinity Il-2 receptor starts to emerge on the cell membrane of activated T cells, and after the binding of Il-2 to the receptor, the cells enter the proliferation cycle. The biological effects of Il-2, although originally defined on the basis of the T-cell growth, have expanded to include interactions with macrophages, activated B cells, NK-cells and other cytotoxic cells. It is a very important cytokine with a central role in a specific immune response to antigens (12–14).

Cytokine mediated immune and inflammatory responses can be modulated at two stages: at the activation of cytokine gene with the end consequence of cytokine secretion and at the effector phase involving cytokine-specific receptor interaction (1). From the point of view that the cytokines are natural immune response modifiers, it is very important to know how the regulation of their synthesis and secretion occurs. Especially of interest are the differences between normal and by disease or therapy altered immune system. Due to the complexity of the cytokine network, it is difficult to follow and influence cytokine production in vivo, therefore, in vitro approach to this problem is much more frequently used. Very often this kind of research is performed in mononuclear cell (MNC) cultures with MNC isolated either from peripheral blood or from lymphoid organs. MNC fraction is composed of lymphocytes and macrophages which are main participants in specific immune response to the antigen (15). Usually stimuli other then antigen are used in vitro to mimic natural specific antigen stimulation that occurs in the organism. Cytokine production as part of this process has been intensively studied since the discovery of their existence. T-lymphocytes are known to need at least two distinct stimuli to be activated. Mitogenic lectins deliver the signal through antigen specific T-cell receptor (TCR) which causes the phospholipid turnover, the signal transduction pathway common to many different cell types. With mitogen triggering TCR receptor, the natural activation of T cells with antigen is simulated. On the other hand, phorbol mirystate acetate (PMA) as a ligand of protein kinase C activates this enzyme.

This is a signal transduction pathway from the cell environment to the nucleus specially important for Il-2 production. With PMA we probably replace insufficient costimulatory signals delivered from antigen presenting cells (APC) and essentially needed in T-cell activation for proliferation and expression of effector functions. Monocytes are activated by PMA as a sufficient stimulus, and also by mitogenic lectins, but it is not clear whether they are activated directly or indirectly through activated T-helper cells (16–19).
The aim of our work was to study different responses to various stimuli and the modifications of Il-1 α, Il-1 β and Il-2 production in human peripheral blood MNC cultures according to different cell origins. MNC were isolated from the blood of three groups of donors: healthy volunteers as a source of normal responding MNC, patients with Graves' disease (autoimmune hyperthyreosis – AH) as a source of in vivo hyperactivated MNC and patients after kidney transplantation receiving immunosuppressive therapy (IST) with Cyclosporin A (CsA) and corticosteroides as a source of in vivo suppressed MNC.

Materials and methods

MNC were isolated from the peripheral blood of six healthy volunteers, six patients receiving IST and six patients with AH by the centrifugation on Ficoll-Paque as described elsewhere (20). MNC were counted, calibrated to 1×10^{-6} cells/ml in RPMI 1640 with 10% human AB serum and cultured for 24 hours at 37°C in atmosphere with 5% CO_2 and 95% humidity. Lymphocytes and combination with PMA. Four stimulation modes for each donor's MNC were chosen: 1) with ConA, 2) with PHA, 3) with ConA and PMA simultaneously, and 4) with PHA and PMA simultaneously. Activated MNC in the microcultures secreted cytokines to the cell culture supernatants according to their ability to respond to different activation conditions. After that the supernatants were separated from the cells and kept frozen to await radioimmunoassay (RIA) determination of cytokine concentration.
RIA determinations of cytokine concentrations were performed with RIA kits (Amersham, G. Britain) for Il-1 α and Il-1 β according to the directions for use. Il-2 determination was performed with RIA reagents (Amersham, G. Britain) with a modification of the suggested method. The primary reaction reagents were diluted to 10,000 cpm for labelled Il-2 and to the final volume of 50 ml for Il-2 antibodies. The duration of the primary reaction was 2.45 hours at the temperature of 37°C. Amerlex-M antibodies (Amersham, G. Britain) were used for the primary reaction products separation in reduced amount (250 µl per tube). After the centrifugation, the tubes were placed into the magnetic separator, the supernatants were discarded and the radioactivity was measured.
Statistical parameters i.e. mean value (X), standard deviations (DS), and significant differences between selected groups (Student's t-test) were computed using statistical package Microstat (Ecosoft Inc., USA). Graphs were drawn using scientific graph package Sigmaplot (Jandel Sc., USA).

Results

We compared Il-1 α, Il-1 β and Il-2 production in MNC cultures of three groups of donors. The Figures 1, 2 and 3 show the average production of the three cytokines in one group. Asterisk labels the significant differences in the production of a given cytokine in patient MNC cultures, if compared with normal production using the same mode of stimulation. The results presented by figure 1 show that the production of Il-1 α is affected significantly by lectin mode of stimulation in both groups of patients, but it is completely restituted after combined stimulation in the group of IST patients, and significantly excessive in AH patients. Il-1 β production pattern is similar in both patient groups presented by figure 2, although we could not prove any significant differences because of great individual differences in the production. However, normal Il-1 β production is more PMA-dependent than Il-1 α production. But evident differences between the effects of stimulation modes and between donor groups can be observed in the case of Il-2 production (Fig. 3). It is apparent that healthy MNCs respond to the combined mode of stimulation (PHA and PMA) with massive Il-2 production while it is significantly suppressed in IST patients. AH patients MNC stimulated with PHA and PMA react with a distinctive hyperproduction of Il-2, although the production in PHA stimulation mode is significantly lower if compared with normal production.

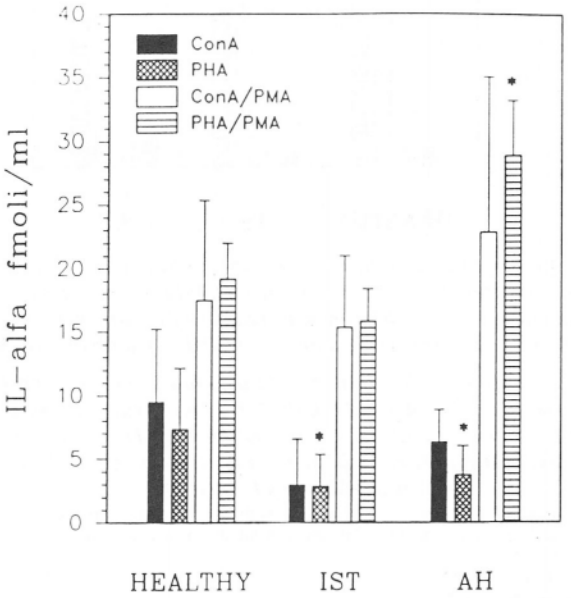

Fig. 1. *Mean values and standard errors (X ± SE) of Il1α (fmol/ml) in MNC cultures of healthy donors (n = 6), IST patients (n = 6) and AH patients (n = 6). MNCs were stimulated on four differents modes: 1. with ConA, 2. with PHA, 3. with ConA&PMA, 4. with PHA&PMA.*

Sl. 1. *Srednje vrednosti in standardne napake (X ± SE) pri izdelovanju Il-1 α (fmol/ml) v kulturah MNC zdravih darovalcev (n = 6), bolnikov z IST (n = 6) in bolnikov z AH (n = 6). MNC smo spodbujali na štiri različne načine: 1. s ConA, 2. s PHA, 3. s ConA&PMA, 4. s PHA&PMA.*

* significant differences in comparison with healthy donors ($p<0.05$)
significantne razlike pri primerjavi z rezultati pri zdravih darovalcih ($p<0,05$)
ConA concanavalin A
konkanavalin A
PHA phytohemagglutinin
fitohemaglutinin
MNC mononuclear cells
mononuklearne celice
AH autoimmune hyperthyreosis (Graves' disease)
avtoimunska hipertiroza
IST immunosuppressive therapy (Cyclosporin A and corticosteroids)
imunosupresivna terapija (ciklosporin A in kortikosteroidi)

Discussion

The results of our experiments revealed the fact that the production of cytokines in vitro differs according to different origin of MNC and also to different modes of in vitro stimulation. We could detect not only quantitative differences but also different production patterns of cytokines examined in these experiments. Il-1 is an interesting cytokine appearing in two different forms but their relationship to the role of Il-1 in immune and inflammatory

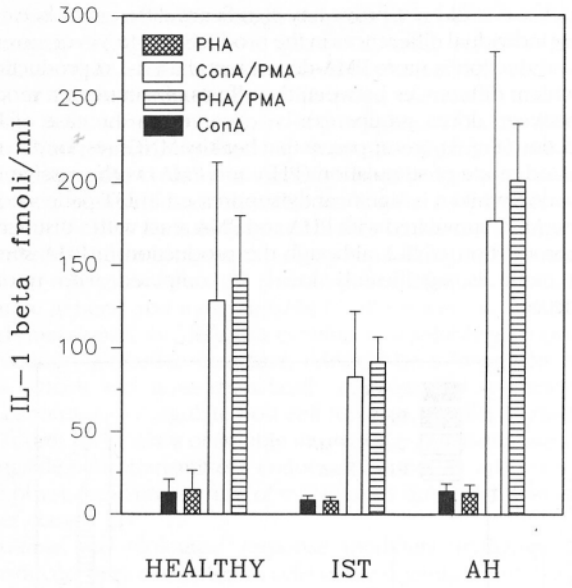

Fig. 2. *Mean values and standard errors (X ± SE) of Il-1β (fmol/ml) in MNC cultures of healthy donors (n = 6), IST patients (n = 6) and AH patients (n = 6). MNC were stimulated in four different modes: 1. with ConA, 2. withPHA, 3. with ConA&PMA, 4. with PHA&PMA.*

Sl. 2. *Srednje vrednosti in standardne napake (X ± SE) pri izdelovanju Il-1β (fmol/ml) v kulturah MNC zdravih darovalcev (n = 6), bolnikov z IST (n = 6) in bolnikov z AH (n = 6). MNC smo spodbujali na štiri različne načine: 1. s ConA, 2. s PHA, 3. s ConA&PMA, 4. s PHA&PMA.*

* significant differences in comparison with healthy donors (p<0.05)
signifikantne razlike pri primerjavi z rezultati pri zdravih darovalcih (p<0,05)
ConA concanavalin A
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PHA phytohemagglutinin
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MNC mononuclear cells
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AH autoimmune hyperthyreosis (Graves' disease)
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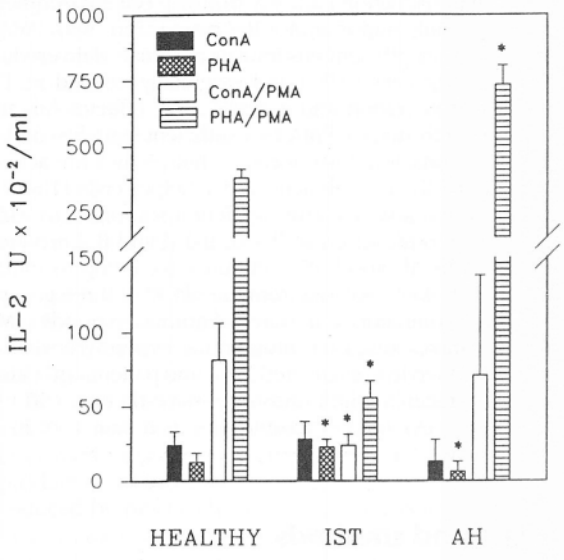

Fig. 3. *Mean values and standard errors (X ± SE) of Il-2 U × 10^{-2}/ml) in MNC cultures of healthy donors (n = 6), IST patients (n = 6) and AH patients (n = 6). MNC were stimulated in four different modes: 1. with ConA, 2. with PHA, 3. with ConA&PMA, 4. with PHA&OMA.*

Sl. 3. *Srednje vrednosti in standardne napake (X ± SE) pri izdelovanju Il-2 U × 10^{-2}/ml) v kulturah MNC zdravih darovalcev (n = 6), bolnikov z IST (n = 6) in bolnikov z AH (n = 6). MNC smo spodbujali na štiri različne načine: 1. s ConA, 2. s PHA, 3. s ConA&PMA, 4. s PHA&PMA.*

* significant differences in comparison with healthy donors (p<0.05)
signifikantne razlike pri primerjavi z rezultati pri zdravih darovalcih (p<0,05)
ConA concanavalin A
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PHA phytohemagglutinin
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MNC mononuclear cells
mononuklearne celice
AH autoimmune hyperthyreosis (Graves' disease)
avtoimunska hipertiroza
IST immunosuppressive therapy (Cyclosporin A and corticosteroids)
imunosupresivna terapija (ciklosporin A in kortikosteroidi)

responses has not been clarified yet (11, 21, 22). Our results show that the synthesis of Il-1 α and Il-1 β is regulated differently.

Il-1 α production is stimulated by lectins and PMA costimulation shows additive effects in the cultures of healthy MNC. In the case of IST patients, we observe a strong lectin-dependent production suppression. We could explain this with the stimulation of macrophages by lectins via activated T cell, in our case strongly suppressed by the CsA. PMA directly triggers macrophages, which seem to respond normally. The same pattern is observed in AH patients, which is surprisin: normal or hyperproduction as seen in combined stimulation was expected.

In the case of Il-1 β, the addition of PMA acts more synergistically on the production in the group of healthy donors when compared with Il-1 α production. But in the two groups of patients we could not prove significant differences in the production because of individually greatly varying production, which is reported also by other authors (23). From this point of view Il-1 α seems to be a more appropriate Il-1 in vitro production marker.

Distinct differences observed in the case of Il-2 in vitro production in MNC cultures of three origins are consistent with the current knowledge about mechanisms of T-cell activation and drug-induced immunosuppression. It is evident that the T cell requires two pathways of activation, e.g. lectin stimulation, which triggers T-cell receptor and imitates antigen binding to this receptor, and PMA costimulation, which activates protein kinase C pathway and probably imitates the coactivation of T cell through accessory molecules on the antigen-presenting cell membrane (17, 24).

But on the other hand, there are some new data revealed by our results which elucidate probably hyperactive T cell in autoimmune disease. In the case of PHA stimulation of AH patients MNC Il-2 production was significantly lower when compared with "healthy" production. This so called "peripheral" Il-2 defect was already described in some other autoimmune diseases, and it was explained with the exhaustion of T cells in vivo (25). But in our opinion the results obtained with the combined stimulation are more relevant because the situation in vivo, with an excess of APC, is simulated more closely. On the other hand, the combined stimulation reveals the ability of T-cells for hyperproduction of Il-2, which has not yet been described in association with human autoimmune diseases. Two interpretations of this finding are possible: Il-2 is being hyperproduced either during the progression of the disease and is involved in selfamplifying processes, or Il-2 hyperproduction precedes the autoimmune disease and can be involved in abrogation of peripheral tolerance to autoantigens via breaking of clonal anergy and activation of autoreactive T-cell clones. This means the beginning of autoaggression and disease development.

References

1. Mosmann TR. Cytokines: is there biological meaning? Curr Opin Immunol 1991; 3: 311–4.
2. Nathan C, Sporn M. Cytokines in context. J Cell Biol 1991; 113: 981–6.
3. Arai K, Lee F, Miyajima A, Miyatake S, Arai N, Yokota T. Cytokines: Coordinators of immune and inflammatory responses. Annu Rev Biochem 1990; 59: 783–836.
4. Swain SL. Lymphokines and the immune response: the central role of interleukin-2. Curr Opin Immunol 1991; 3: 304–10.
5. Oppenheim JJ, Sevach EM. Immunophysiology. New York, Oxford: Oxford University Press, 1990: 210–25.
6. Aarden LA, Brunner TK, Cerotti JC et al. Revised nomenclature for antigen-nonspecific proliferation and helper factors. J Immunol 1979; 123: 2928–9.
7. WHO-IUIS nomenclature subcommitee in interleukin designation. Nomenclature for secreted regulatory proteins of the immune system (interleukins). Bull Word Health Organ 1991; 69: 483–4.
8. Zurakowski G, de Vries JE. Interleukin-13, an interleukin 4-like cytokine that acts on monocytes and B cells, but not on T cells. Immunol Today 1994; 15: 19–26.
9. Gery I, Davies P, Derr J, Krett N, Barranger JA. Relationship between production and release of lymphocyte-activating factor (interleukin-1 by murine macrophages. I. Effects of various agents. Cell Immunol 1981; 64: 293–303.
10. Di Giovine FS, Duff GW. Interleukin 1: the first interleukin. Immunol Today 1990; 11: 13–20.
11. Dinarello CA. Interleukin-1 and interleukin-1 antagonism. Blood 1991; 77: 1627–52.
12. Gillis S, Ferm M, Ou W, Smith K. T cell growth factor: parameters of production and quantitative microassay for activity. J Immunol 1978; 120: 2027–32.
13. Ruscetti FW. Interleukin-2. In: Immunophysiology. New York, Oxford: Oxford University Press, 1990: 46–66.
14. Smith KA. Interleukin-2. Curr Opinion Immunol 1992; 4: 271–6.
15. Klein J. Defence reactins mediated by phagocytes. In: Immunology, Boston, Oxford, London, Edinbourgh, Melbourne: Blackwell Scientific Publications, 1990: 311–334.
16. Nishizuka Y. The role of protin kinase C in cell surface signal transduction and tumour promotion. Nature 1984; 308: 693–8.
17. Berry N, Ase K, Kikkawa U, Kishimoto A, Nishizuka Y. Human T cell activation by phorbol esters and diacylgycerol analogues. J Immunol 1989; 143: 1407–13.
18. Janeway CA, Goldstein P. Lymphocyte activation and effector functions. Curr Opin Immunol 1992; 4: 241–5.
19. Jenkins MK, Johnson JG. Molecules involved in T-cell costimulation. Curr Opin Immunol 1993; 5: 361–7.
20. Sollner M, Kotnik V, Pečar S, Štalc A, Simčič S, Povšič L, Herzog-Wraber B, Klampfer L, Ihan A, Grosman P. Apyrogenic synthetic desmuramylpeptide LK-409, with immunomodulatory properties. Agents Actions 1993; 38: 273–80.
21. Durum SK, Oppenheim JJ, Neta R. Immunophysiologic role of interleukin 1. In: Immunophysiology. New York, Oxford: Oxford University Press, 1990.
22. Oppenheim JJ, Ruscetti FW, Faltynek C. Cytokines. In: Basical and clinical immunology. London, Sydney, Toronto: Appelton&Lange 1991: 78–100.
23. Goode HF, Rathbone BJ, Kelleher J, Walker BE. Monocyte zinc and in vitro prostaglandin E_2 and interleukin 1 beta production by cultured peripheral blood monocytes in patients with Chron' disease. Digestive Dis Sci 1991; 36: 627–33.
24. Janeway CA, Goldstein P. Lymphocyte activation and effector functions. Curr Opin Immunol 1993; 5: 313–23.
25. Kroemer G, Anderu-Sanchez JL, Gonzalo JA, Gutierrez-Ramoz JC, Martinez AC. Interleukin-2, autotolerance, and autoimmunity. Adv Immunol 1991; 150: 147–235.

Professional article/Strokovni prispevek

MODULATION OF NEUTROPHIL OXIDATIVE BURST

MODULACIJA OKSIDATIVNE EKSPLOZIJE NEVTROFILCEV

Saša Simčič[1], Fedja Bobanović[2]

[1] Institute of Microbiology, Medical Faculty, Zaloška 4, 61000 Ljubljana, Slovenia
[2] Faculty of Electrical and Computer Engineering, University of Ljubljana, Tržaška 25, 61000 Ljubljana, Slovenia

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Key words: *activation; membrane potential; pulsed electric current; calcium; chronic granulomatous disease*

Abstract – Background. *The neutrophil activation pathways include complex changes in ionic conductances and membrane potential. These changes are considered to be early parameters of the neutrophil activation process, however, their exact functional role is still unclear. In our study, an external electric field that changes the neutrophil membrane potential was used to assess the correlation of the membrane potential changes to the oxidative burst (OB) response.*

Materials and methods. *Venous blood was taken from healthy adults and from the patients with chronic granulomatous disease (CGD) without any other manipulation to estimate the neutrophil activation measured with luminol-enhanced chemiluminescence (CL). Phorbol myristate acetate (PMA), chemotactic peptide N-formylmethionylleucylphenylalanine (fMLP) and calcium ionophore ionomycin were used for in vitro activation of the OB. For electric treatment, the PEC was applied for one or five minutes through Pt-Ir electrodes directly immersed in cell suspension. The time course of the OB response or the CL levels before and after treatment were chosen as the main estimation values. The statistical significance was analysed by a paired Student's t-test.*

Results. *The PEC treatment induced no significant OB response in normal human neutrophils. The one-minute PEC treatment affected mostly the ionomycin- and fMLP-stimulated PMNs and less PMA-stimulated PMNs. The CGD PMNs exposed to either five-minute PMA, PEC or combined PMA and PEC treatment showed no significant alteration in CL levels compared to nonexposed control PMNs. The CL levels of normal PMNs stimulated for five minutes with combined PMA and PEC were 53% higher than in PMA-alone-activated neutrophils. However, the CL level of PMA-activated neutrophils in calcium depleted medium was only half as high as in normal calcium medium. Five-minute activation of the OB with PMA and PEC in calcium depleted medium was 13% higher than in solely PMA-treated PMNs.*

Conclusions. *PEC affects stimulated response in normal PMNs. A close association between membrane potential changes and OB was demonstrated, however, these changes are not a trigger in signal transduction, since there were no significant changes in the neutrophil CL level after PEC treatment. The PEC preferentially affects calcium-dependent activation with ionomycin and fMLP rather than activation with PMA where calcium is needed but not absolutely necessary for OB response. The PEC effect is closely related to cellular calcium mobilisation, since depletion of extracellular calcium highly suppressed the PEC effect.*

Ključne besede: *aktivacija; membranski potencial; pulzni električni tok; kalcij; kronična granulomatoza*

Izvleček – Izhodišča. *Aktivacijske poti nevtrofilca vključujejo obsežne spremembe ionskih prevodnosti in membranskega potenciala. Te spremembe so zgodnji parametri aktivacije nevtrofilca, vendar njihova funkcijska vloga še ni pojasnjena. Ker je znano, kako se spremeni membranski potencial okrogle celice, kot je nevtrofilc v zunanjem električnem polju, smo uporabili pulzni električni tok, da bi ocenili povezavo sprememb membranskega potenciala z oksidativno eksplozijo.*

Materiali in metode. *Vensko kri zdravih odraslih in bolnikov s kronično granulomatozo brez druge obdelave smo uporabili za ocenitev aktivacije nevtrofilca, ki smo jo merili z luminolom ojačano kemiluminiscenco (CL). Za in vitro aktivacijo oksidativne eksplozije (OB) smo uporabili forbolni miristat acetat (PMA), kemotaktični peptid N-formilmetionillevcilfenilalanin (fMLP) in kalcijev ionofor ionomicin. Za tretiranje s tokom smo uporabili eno- ali petminutni tretma s pulznim električnim tokom (PEC), ki smo ga privedli s Pt-Ir elektrodami, neposredno potopljenimi v suspenzijo celic. Za ovrednotenje aktivacije smo si izbrali časovni potek oksidativne eksplozije in raven kemiluminiscence pred in po tretiranju. Statistično značilnost razlik smo izračunali s parnim t-testom po Studentu.*

Rezultati. *Tretiranje s pulznim električnim tokom ni izzvalo oksidativne eksplozije v normalnih humanih nevtrofilcih. Enominutno tretiranje je vplivalo najbolj na nevtrofilce, stimulirane z ionomicinom in fMLP, manj pa na s PMA stimulirane nevtrofilce. Nevtrofilci bolnikov s kronično granulomatozo, izpostavljeni petminutnemu tretiranju s PMA, PEC, ali PMA in PEC se niso odzvali z nobenimi značilnimi spremembami kemiluminiscence v primerjavi z neizpostavljenimi kontrolnimi celicami. Raven kemiluminiscence normalnih nevtrofilcev, stimuliranih pet minut s kombinacijo PMA in PEC, je bila 53% višja, kot je bila raven kemiluminiscence samo s PMA aktiviranih nevtrofilcev. Kemiluminiscenca s PMA aktiviranih nevtrofilcev v mediju, kjer kalcij ni bil navzoč, je bila samo polovica tiste, kjer so bili nevtrofilci v mediju s fiziološko koncentracijo kalcija. Petminutna aktivacija oksidativne eksplozije s PMA in PEC v mediju, kjer kalcij ni bil navzoč, pa je bila samo 13% višja kot pri samo s PMA tretiranih nevtrofilcih.*

Zaključki. *Pulzni električni tok vpliva na spodbujen odziv v normalnih nevtrofilcih. Dokazana je bila tesna povezava med spremembami membranskega potenciala in oksidativno eksplozijo, vendar pa te spremembe verjetno niso sprožilec v prenosu signala, ker nismo opazili značilnih razlik v kemiluminiscenci po tretiranju z električnim tokom. Pulzni električni tok prednostno prizadene od kalcija odvisno aktivacijo z ionomicinom in fMLP, manj pa aktivacijo s PMA, kjer je kalcij sicer potreben, ne pa zahtevan za oksidativno eksplozijo. Efekt pulznega električnega toka je tesno povezan z mobilizacijo celičnega kalcija, ker je odstranitev zunajceličnega kalcija zelo zmanjšala vpliv toka.*

Introduction

Polymorphonuclear leukocytes (PMNs, neutrophils) play a major role in the body's defence against harmful foreign pathogens, as evidenced by the often life-threatening infections acquired by patients with chronic granulomatous disease (CGD), neutropenia, leukemia, or congenital diseases affecting leukocyte structure and function (1). In order to perform this role. PMNs migrate from the circulation to sites of tissue damage or inflammation under the influence of chemoattractant factors produced by various humoral or cellular immunologic processes. Upon reaching those sites, PMNs phagocytoze and destroy microorganisms and damaged tissue with an array of microbicidal oxidants, proteolytic enzymes, and antimicrobial peptides. Under certain circumstances, the excessive or inappropriate release of these highly destructive agents can result in undesirable tissue damage, therefore, PMNs can also be considered the primary cellular mediators of pathologic inflammation (2).

The process of neutrophil activation involves a variety of cellular responses, including cell shape changes, aggregation, phagocytosis, granule enzyme secretion, and stimulation of the oxidative burst (OB). The OB is mediated by a membrane-bound NADPH oxidase, which generates superoxide anion (O_2^-), the precursor of powerful microbicidal oxidants (2). All of these processes are dependent on a series of distinct events: (1) the binding of chemoattractant ligands by specific cell surface receptors, (2) the transduction of the binding event signal into an intracellular signal for cell activation, and (3) the stimulation (or inhibition) of the appropriate biochemical pathways that lead to neutrophil activation.

Biochemical pathways of polymorphonuclear leukocyte activation

The stimulus-response pathways operating in the neutrophil OB have not been completely elucidated yet. One pathway may involve the hydrolysis of phosphatidylinositol bisphosphate (PIP_2) by a receptor-linked phospholipase C (PLC) to give inositol trisphosphate (IP_3), which mobilises intracellular calcium and diacylglycerol (DAG). Both activate protein kinase C (PKC), which has an important role in stimulating superoxide production by the neutrophil (3, 4). Recent evidence has indicated that inositol lipid hydrolysis is not the only source of DAG in activated cells. A number of agonists elicit functional responses in the absence of IP_3 formation, or an increase in intracellular calcium (5). Furthermore, many of these responses are accompanied by raised DAG levels or increased phosphatidylcholine turnover (5). Tumor-promoting phorbol ester, phorbol myristate acetate (PMA) may substitute for DAG, and it activates PKC directly in the first phase which is followed by the activation of NADPH oxidase assembly (6), but it does not activate phosphoinositide-specific PLC (5). However, the subsequent phase of DAG production, which may be required to sustain the OB, is mediated by the action of a phospholipase D on phosphatidylcholine coupled with phosphatidate phosphatase. Furthermore, a large part of the DAG formed in chemotactic peptide N-formylmethionylleucylphenylalanine (fMLP) stimulated PMNs results from the activation of the receptor-linked phospholipase D (5). The operation of these pathways, which indicates the increased DAG levels, however derived, and subsequent PKC activation are involved in the OB response.

Ion transport and membrane potential changes during activation in polymorphonuclear leukocyte

The activation pathways include complex changes in ion fluxes across membranes, alterations in the intracellular concentrations of ions, and changes in the binding of ions to membrane and intracellular components (7, 8). The early events that follow the binding of ligand to the membrane include changes in membrane potential (Vm) and intracellular pH. A close association between membrane depolarization and the OB in normal neutrophils was demonstrated (8). In patients with CGD, a clinical syndrome of severe and recurrent infections that is biochemically characterised by the absence of the neutrophil OB, the membrane potential changes after cell stimulation were not observed (9).

Intracellular Ca^{2+} concentrations ($[Ca^{2+}]i$) are thought to have a major role in the regulation of cellular responses at different stages. Increases in $[Ca^{2+}]i$ have a dual origin: mobilisation from intracellular stores, the so-called IP_3-sensitive calciosomes, and influx from the extracellular medium (11). In neutrophils, Ca^{2+} acts in at least three ways: first, some pool of Ca^{2+} maintains receptor expression; second, some pools of $[Ca^{2+}]i$ take part in signal transduction of different stimuli, and third, $[Ca^{2+}]i$ transients potentiate, but are not required for function (10). PMNs in which $[Ca^{2+}]i$ is driven to high levels by ionophores A23187 or ionomycin, mobile ion carriers for Ca^{2+}, promptly degranulate and make OB. Great attention has been devoted to the feedback control of $[Ca^{2+}]i$. It has become increasingly clear that Ca^{2+}-activated enzyme PKC plays a dual role in the regulation of neutrophil responses: stimulatory and inhibitory. Stimulation by PMA induces several PMN responses without increasing $[Ca^{2+}]i$. PKC feedback regulation is exerted at different levels: (1) PKC increases the activity of the plasma membrane Ca^{2+}-ATP ase (2) by causing depolarization of Vm, which reduces IP_3 generation and therefore mobilisation of Ca^{2+}, and (3) by acting on PLC. Another important feedback mechanism involves changes in the intracellular concentrations of cAMP, which acts directly by inhibiting increases in $[Ca^{2+}]i$ or by activating protein kinase A followed by phosphorylation of a receptor (12).

Neutrophils are not electrically excitable cells, however, it is known that changes in membrane potential (Vm) are involved in their activation. The exact functional role of Vm changes is still unclear. The external electric field induces a position-dependent modulation of the membrane potential difference which is superimposed on the resting potential (13). Therefore, we might expect that an external electric field that change the resting membrane potential of the neutrophil affects the neutrophil activation.

In our study, the pulsed electric current/field (PEC) was used, alone or combined with PMA, fMLP or ionomycin (chemical stimuli which operate through distinctly different activation pathways) to assess a correlation of Vm changes to the OB response. Experiments on normal human PMNs and PMNs of CGD patients were performed. Luminol-enhanced chemiluminescence (CL), which is an effective analytical technique for the estimation of the OB response of phagocytic cells, was used (14).

Materials and methods

Chemicals

PMA, fMLP, ionomycin, luminol (5-amino-2, 3-dihydro-4-phthalazinedione), Hank's balanced salt solution (HBSS) and dimethyl sulfoxide (DMSO) were obtained from Sigma (St. Louis, USA). The chemicals for modified HBSS (HBSS-EGTA), i.e. Ca^{2+}-free HBSS with 3 mM ethylene glycol-bis [ß-aminoethyl ether]-N, N, N' N'-tetraacetic acid (EGTA), were obtained from Merck (Darmstadt, Germany). PMA, fMLP, ionomycin and luminol were dissolved in DMSO and added to the cell suspension to give a final concentration of 3 µM PMA, 1.5 µM fMLP or ionomycin, 10 µM luminol and 0.1% DMSO (v/v) in HBSS or HBSS-EGTA.

Blood samples

Venous blood (5 ml) was taken from healthy adults (18–45 years old) into heparinized (50 IU) tubes. Their hematological characteristics, such as the leukocyte count (Coulter Counter, USA), the differential count and the erythrocyte sedimentation rate, were

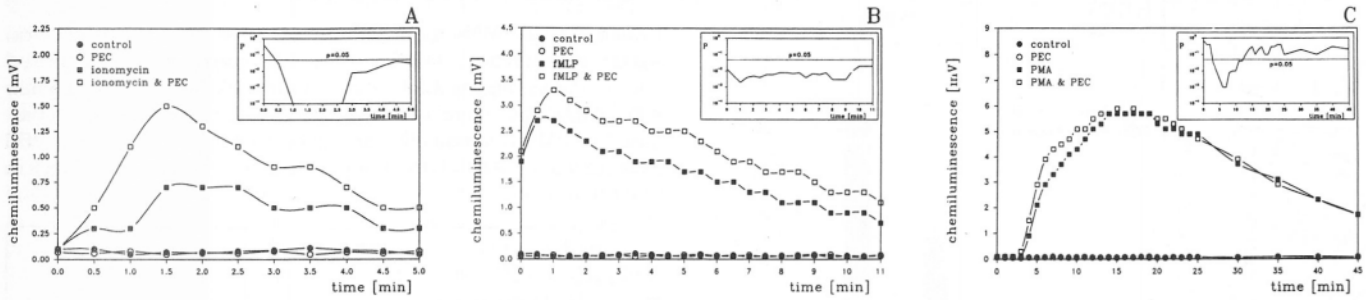

Fig. 1. *The one-minute treatment effect of 20 mA amplitude PEC, ionomycin, fMLP, PMA, and combinations of them on the time course of the neutrophil OB measured with chemiluminiscence. Each point represents the mean of 7 samples. The statistical significance was analysed by a paired Student's t-test. (A) The effect of PEC, 1.5 μM ionomycin and their combination on the OB. (B) The effect of PEC, 1.5 μM fMLP and their combination on the OB. (C) The effect of PEC, 3 μM of PMA and their combination on the OB.*

Sl. 1. Vpliv enominutnega tretiranja s pulznim električnim tokom, amplitude 20 mA, ionomicinom, fMLP, PMA in njihovimi kombinacijami na časovni potek oksidativne eksplozije nevtrofilca, merjene s kemiluminiscenco. Vsaka točka predstavlja povprečje sedmih vzorcev. Statistično značilnost smo izračunali s parnim testom po Studentu. (A) Vpliv pulznega električnega toka, 1,5 μM ionomicina in njune kombinacije na oksidativno eksplozijo. (B) Vpliv pulznega električnega toka, 1,5 μM fMLP in njune kombinacije na oksidativno eksplozijo. (C) Vpliv pulznega električnega toka, 3 μM PMA in njune kombinacije na oksidativno eksplozijo.

within normal range (15). For certain experiments blood was obtained from two patients with CGD of childhood. Samples were used to assess neutrophil activation without any other manipulation. At the beginning, 200 μl of heparinised blood was resuspended in 400 μl HBSS, or in some experiments, in HBSS-EGTA. With regard to activation treatment, blood samples were divided into four groups: control, PEC, chemical stimulant (PMA, fMLP or ionomycin) and chemical stimulant & PEC. The samples in the control groups were treated neither with chemical stimulants nor with PEC. In the PEC groups, samples were treated with electric current alone. In the chemical stimulant groups, samples were treated with PMA, fMLP or ionomycin solution. In the chemical stimulant & PEC groups, samples were treated with PMA, fMLP or ionomycin and electric current. Experimental groups consisted of 7, 10 or 20 samples, as indicated. During treatment, samples of all groups were shaken in cuvettes with electrodes at room temperature.

Neutrophil OB activation

A 3 μM final concentration of PMA and 1.5 μM final concentration of fMLP or ionomycin in HBSS were used for in vitro activation of OB. In some experiments, 3 μM final concentration of PMA in HBSS-EGTA was used. For electric treatment, four-second trains of biphasic, asymmetrical, charge balanced pulses, separated by four-second pauses were used; the pulse amplitude was 20 mA, frequency 40 Hz, and the pulse duration 0.25 ms. PEC was applied for one minute or 5 min through Pt-Ir (90/10%) electrodes directly immersed in suspension. During treatment, signals were monitored by current probe/oscilloscope combination (Tektronix P6042/7704, USA). All hematological parameters listed above were checked after treatment with PEC. Not even 60 min of PEC caused any significant changes.

Chemiluminescence (CL) assay

CL was measured on a LKB-Wallac 1250 luminometer in polystyrene measuring cuvettes (Clinicon, Finland). After particular activation, or control treatment, 400 μl of luminol solution in HBSS or HBSS-EGTA were added to each cuvette. The cuvettes were then transferred into the counting chamber and the results expressed in mV are the mean values of measurements lasting for ten seconds. The time course of the OB response or the CL levels before and after 5-min treatment were chosen as the main estimation values. The statistical significance of the results was analysed by a paired Student's t-test.

Results

PMA-, fMLP- and ionomycin-stimulated neutrophils

Control and one-minute PEC treatment induced no significant OB response (Fig. 1: a, b, c). The PEC did not change the OB response curves for any of the three OB activators in normal human neutrophil. The one-minute PEC treatment preferentially affected the ionomycin-stimulated neutrophil, rather than fMLP- or PMA-stimulated one. The integrated OB response (an area under a response curve) of the ionomycin & PEC-stimulated cells was 87% higher than in solely ionomycin-treated cells, whereas the peak CL was 114% higher (Fig. 1a). The integrated OB response of the fMLP&PEC-activated neutrophils was 32% higher than in the fMLP-treated cells (Fig. 1b) and in the PMA&PEC-treated neutrophils only 4.7% higher than in the PMA-alone-treated cells (Fig. 1c). The peak CL after combined fMLP&PEC treatment was 22% higher compared to the fMLP-alone-activated cells. However, there were no significant differences in the peak CL between the PMA&PEC- and the PMA-treated neutrophils. The statistical significance of the results is shown on the Figures.

Stimulation of neutrophils in normal and Ca^{2+}-depleted medium

Using the same concentration of PMA (3μM) in both presence and absence of extracellular Ca^{2+}, the CL response levels after 5-min treatment with either PMA or PMA&PEC, were measured. The control and the PEC treatment caused no significant changes in PMN CL levels in HBSS (Fig. 2) as well as in HBSS-EGTA (Fig. 3). In the PMA&PEC group, the CL levels in HBSS were (53±7)% (mean±S.E.; n=20) higher than in the PMA group. The difference was statistically significant at a high level of confidence ($P<0.0003$). However, the CL levels of PMA-activated cells in HBSS-EGTA were (48±3)% of the CL levels in the PMA group in HBSS. The PEC effect in HBSS-EGTA was highly reduced, namely the CL levels in the PMA&PEC group were only (13±3)% higher than in the PMA group. The difference was still highly significant ($P<0.0005$).

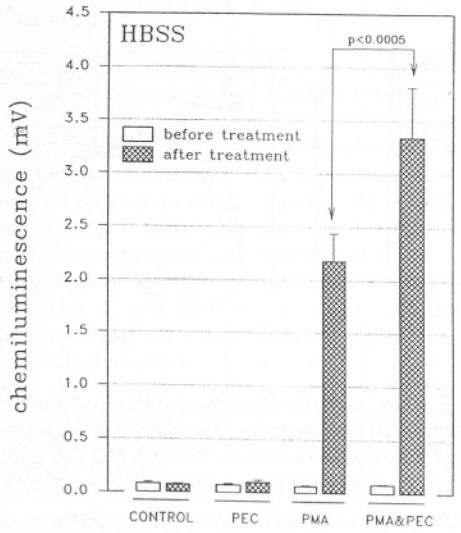

Fig. 2. *The 5-min treatment effect of 20 mA amplitude PEC, 3 μM PMA and a combination of them on neutrophil OB measured with chemiluminiscence in HBSS. The results are mean ± S.E. of 20 samples. The difference between PMA and PMA & PEC treatment was shown to be statistically significant by a paired Student's t-test.*

Sl. 2. *Petminutni vpliv tretiranja s pulznim električnim tokom, amplitude 20 mA, 3 μM PMA in njune kombinacije na oksidativno eksplozijo nevtrofilca, merjene s kemiluminiscenco v HBSS. Rezultati so povprečna vrednost ± standardna napaka 20 vzorcev. Razlika med tretmajem s PMA in PMA in pulzivnim električnim tokom je statistično značilna, izračunana s parnim t-testom po Studentu.*

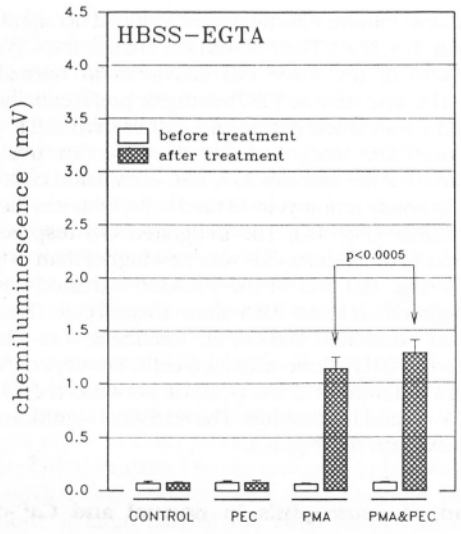

Fig. 3. *The 5-min treatment effect of 20 mA amplitude PEC, 3 μM PMA and a combination of them, on neutrophil OB measured with chemiluminiscence in HBSS-EGTA. The results are mean ± S.E. of 20 samples. The difference between PMA and PMA & PEC treatment was shown to be statistically significant by a paired Student's t-test.*

Sl. 3. *Petminutni vpliv tretiranja s pulznim električnim tokom, amplitude 20 mA, 3 μM PMA in njune kombinacije na oksidativno eksplozijo nevtrofilca, merjene s kemiluminiscenco v HBSS-EGTA. Rezultati so povprečna vrednost ± standardna napaka 20 vzorcev. Razlika med tretmajem s PMA in PMA in pulznim električnim tokom je statistično značilna, izračunana s parnim t-testom po Studentu.*

PEC treatment of CGD neutrophils

Unlike normal PMNs, the CGD PMNs exposed to PMA showed no significant alteration of CL levels (n=10) compared to nonexposed control PMNs (Fig. 4). CL levels of normal PMNs challenged 5 min with PMA&PEC were (51±8)% (mean±S.E.; n=10) higher compared to PMA treated cells. However, five minutes of either PEC treatment or PMA&PEC treatment caused no significant changes in CGD neutrophils activity.

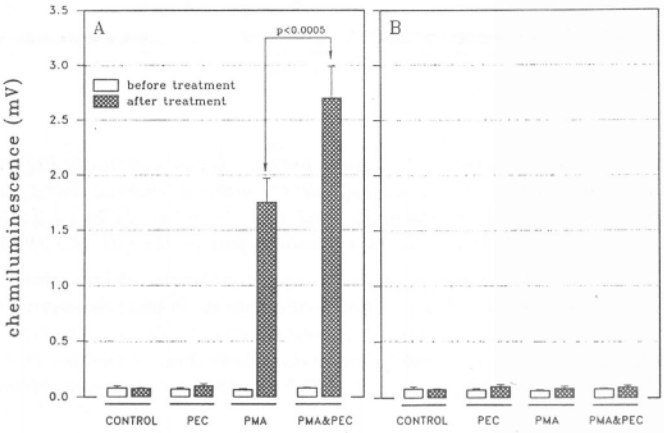

Fig. 4. *The 5-min treatment effect of 20 mA amplitude PEC, 3 μM PMA and a combination of them, on normal (A) and CGD patients' (B) neutrophil OB measured with chemiluminiscence. The results are mean ± S.E. of 10 samples. The difference between PMA and PMA & PEC treatment was shown to be statistically significant by a paired Student's t-test.*

Sl. 4. *Petminutni vpliv tretiranja s pulznim električnim tokom, amplitude 20 mA, 3 μM PMA in njune kombinacije na oksidativno eksplozijo normalnega nevtrofilca (A) in nevtrofilca bolnikov s kronično granulomatozo (B), merjene s kemiluminiscenco. Rezultati so povprečna vrednost ± standardna napaka 10 vzorcev. Razlika med tretmajem s PMA in PMA in pulznim električnim tokom je statistično značilna, izračunana s parnim t-testom po Studentu.*

Discussion

Despite considerable progress in understanding of the biochemical signalling pathways in neutrophils parallel to the progress in describing ionic events, a myriad of questions remains. The most relevant is the relationship of membrane potential, ion fluxes, intracellular Ca^{2+} and pH to neutrophil function. We have examined the effect of the PEC on the OB response. Our results suggest that membrane potential changes in human neutrophils do not elicit the response by itself, thus they are not a trigger of the signalling as in electrically excitable muscle or nerve cells. On the other hand, we have demonstrated that Vm changes induced by external electric current modify the OB to different extent when neutrophils are challenged with different chemical stimuli, such as PMA, fMLP or ionomycin. The PEC mostly affects the ionomycin- and the fMLP-stimulated OB and less the PMA-stimulated OB response, which suggests that Vm changes and ion transport produced by the PEC may not be important in all signalling pathways, however, the exact mechanisms of PMN response to the PEC remain to be determined.

It is generally accepted that both, the intact membrane and soluble cytoplasmic proteins of the NADPHoxidase assembly are necessary for optimal one-electron transfer from NADH to oxygen (16). Since CGD PMNs are incapable of producing the OB, the predictable absence of the PMA-stimulated OB response in our experiment with CGD neutrophils has been observed (17). Also, the absence to initiate normal changes in membrane permeability followed by the biphasic potential shifts in CGD neutrophils after

particular stimulation was described by Castranovat et al. (9). Similarly, our observations that the PEC, either alone or with PMA, failed to induce the OB in CGD PMNs support the view that CGD is linked to a general membrane defect where oxidase may have a major role as proposed by recent investigations (1, 16).

Our results indicate that in neutrophil oxidative burst model, the PEC effect is closely related to cellular calcium mobilisation from extracellular space (18), since depletion of extracellular calcium highly suppressed the PEC effect.

In conclusion, the results of the present study suggest that the PEC probably modulates the OB response in the neutrophil and that this modulation is mostly related to calcium influx from extracellular space. Nevertheless, further experiments should be done to determine the exact regulatory pathways of the PEC.

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References

1. Gallin JI. Disorders of phagocytic cells. In: Gallin JI, Goldstein IM, Snyderman R eds. Inflammation: Basic principles and clinical correlates. New York: Raven Press, 1992: 859-74.
2. Klebanoff SJ. Oxygen metabolites from phagocytes. In: Gallin JI, Goldstein IM, Snyderman R eds. Inflammation: Basic principles and clinical correlates. New York: Raven Press, 1992: 541-88.
3. Sha'afi RI, Molski TFP. Activation of the neutrophil. Prog Allergy 1988; 42: 1–64.
4. Traynor-Kaplan AE. Phosphoinositide metabolism during phagocytic cell activation. In: Kleinzeller A, Grinstein S, Rotstein OD eds. Mechanisms of leukocyte activation. San Diego: Academic Press, 1990: 303–32.
5. Hardy SJ, Robinson BS, Poulos A, Harvey DP, Ferrante A, Murray AW. The neutrophil respiratory burst. Eur J Biochem 1991; 198: 801–6.
6. Nishizuka Y. The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature 1984; 308: 693–8.
7. Gallin EK, Mckinney LC. Monovalent ion transport and membrane potential changes during activation in phagocytic leukocytes. In: Kleinzeller A, Grinstein S, Rotstein OD eds. Mechanisms of leukocyte activation. San Diego: Academic Press, 1990: 127–52.
8. Gallin EK. Ion channels in leukocytes. Physiol Rev 1991; 71: 775–811.
9. Castranova V, Jones GS, Phillips RM, Peden D, Vandyke K. Abnormal responses of granulocytes in chronic granulomatous disease. Biochim Biophys Acta 1981; 645: 49–53.
10. O'Flaherty JT, Rossi AG, Jacobson DP, Redman JF. Roles of Ca in human neutrophil responses to receptor agonists. Biochem J 1991; 278: 705–11.
11. DiVirgilio F, Stendahl O, Pittet D, Lew PD, Pozzan T. Cytoplasmic calcium in phagocyte activation. In: Kleinzeller A, Grinstein S, Rotstein OD eds. Mechanisms of Leukocyte Activation. San Diego: Academic Press, 1990: 179–205.
12. Tauber AI, Karnad AB, Ginis I. The role of phosphorylation in phagocyte activation. In: Kleinzeller A, Grinstein S, Rotstein OD eds. Mechanisms of leukocyte activation. San Diego: Academic Press, 1990: 469–95.
13. Grosse C, Schwan HP. Cellular membrane potentials induced by alternating fields. Biophys J 1992; 63: 1632–42.
14. Haynes AP, Fletcher J. Neutrophil function tests. Bailliere's Clin Haematol 1990; 3: 871–87.
15. Bohinjec J. Hematology. Ljubljana: Mladinska knjiga, 1983 (in Slovene).
16. Segal AW. The electron transport chain of the microbicidal oxidase of phagocytic cells and its inolvement in the molecular pathology of chronic granulomatous disease. J Clin Invest 1989; 83: 1785–93.
17. Simčič S, Bobanović F, Kotnik V, Vodovnik L. The pulsed electric current treatment of human neutrophils-comparison of healthy and chronic granulomatous disease (CGD) neutrophils. Period Biol 1993; 95 (1): 121–4.
18. Bobanović F, Simčič S, Kotnik V, Vodovnik L. Pulsed electric current enhances the phorbol ester induced oxidative burst in human neutrophils. FEBS Lett 1992; 311 (2): 95–8.

Research article/Raziskovalni prispevek

IN VIVO INTERFERENCE OF AZITHROMYCIN WITH SOME HUMAN IMMUNE FUNCTIONS

VPLIV AZITROMICINA NA NEKATERE HUMANE IMUNSKE FUNKCIJE IN VIVO

Janez Tomažič[1], Vladimir Kotnik[2], Saša Simčič[2], Branka Wraber[2]

[1] Clinic for Infectious Diseases and Febrile Illnesses, Medical Centre Ljubljana, Japljeva 2, 61105 Ljubljana, Slovenia
[2] Institute of Microbiology, Medical Faculty, Zaloška 4, 61105 Ljubljana, Slovenia

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Key words: *azithromycin; phagocytosis; lymphocytes; mytogens; cytokine receptors*

Ključne besede: *azitromicin; fagocitoza; limfociti; mitogeni; receptorji za citokine*

Abstract – Background. *In vivo effects of a therapeutic dose of azithromycin on some human immune functions were studied thoroughly.*

Methods. *The research was performed as a controlled, double blind clinical trial comparing an azithromycin-treated group (n = 21) and a placebo-treated control group (n = 10). Before and after the administration of azithromycin or placebo (starch), blood samples were taken and the following tests were done: phagocyte microbicidal test with Candida albicans, measurement of oxidative burst in polymorphonuclear leukocytes with luminol amplified chemiluminescence, proliferative lymphocyte responses to stimulation with pokeweed mitogen (PWM) and protein A (Prot A), and the amount of the soluble interleukin-2 receptors (sIL-2R).*

Results. *The study showed that azithromycin had no influence on natural killing mechanisms, but it significantly increased the proliferative lymphocyte response to PWM (p = 0.016). Azithromycin also had no significant influence on lymphocyte response to Prot A, but it significantly elevated the amount of sIL-2R production (p = 0.002).*

Discussion. *Since natural phagocytic killing activity appeared to be intact in azithromycin loaded cells, azithromycin may, together with normal mechanisms of the host's phagocytic cells, help to eradicate intracellular infections caused by sensitive microorganisms. According to the tests performed with PWM, Prot A and to the amount of sIL-2R production, it is likely that azithromycin interferes with interactions between T cells and B cells resulting in stimulation of these cells.*

Conclusion. *Azithromycin is not damaging to the immune system in any way. On the contrary, it is possible that it even increases the activity of the immune system, which gives hope for the patients suffering from all kinds of immune insufficiency.*

Izvleček – Izhodišča. *Za uspešno zdravljenje bakterijskih infekcijskih bolezni je pomembno razumeti odnos med mikroorganizmi, gostiteljevo obrambo in antimikrobnimi zdravili. Imunomodulacija z antibiotiki je sodoben pristop k izboljšanju antimikrobnega zdravljenja. To je posebno pomembno pri bolnikih z okvarjenim imunskim sistemom. Med vsemi antimikrobnimi zdravili makrolidni antibiotiki najbolje vstopajo in se koncentrirajo v celicah. Avtorji so želeli preučiti vpliv terapevtske doze azitromicina na nekatere komponente humanega imunskega sistema in vivo.*

Metode. *Gre za kontroliran klinični preskus, ki primerja testirano azitromicinsko skupino (n = 21) in kontrolno placebo skupino (n = 10). Izbor zdravih preiskovank (starost 20 do 33 let) v obe skupini je bil opravljen po načelu dvojno slepega preskusa. Kriteriji, po katerih so bile preiskovanke uvrščene v študijo, so bili klinični in laboratorijski. Pred in po prejemu azitromicina (500 mg dnevno, 3 dni, oralno) oziroma placeba (škrob) je bila odvzeta kri, v vseh vzorcih pa so bili opravljeni sledeči testi: fagocitno mikrobicidni test s Candida albicans, merjenje oksidativne eksplozije v polimorfonukleusnih levkocitih (PMN) s kemiluminescenčno metodo, proliferativni limfocitni odziv s pokeweed mitogenom (PWM) in proteinom A (Prot A) in količina topnih receptorjev za interlevkin 2 (tIL-2R) v kulturah mononukleusnih celic, spodbujenih s fitohemaglutininom in forbolnim miristat acetatom.*

Rezultati. *Azitromicin ni signifikantno vplival na fagocitno mikrobicidne mehanizme fagocitov, kakor tudi ne na oksidativno eksplozijo v PMN periferne venske krvi. Signifikantno pa je povečal proliferativni odziv limfocitov s PWM (p = 0.016). Proliferativni odziv limfocitov s Prot A ni bil signifikanten. Azitromicin je signifikantno povečal tudi tvorbo tIL-2R v kulturah MNC (p = 0.002).*

Razpravljanje. *Azitromicin ne vpliva na fagocitne mikrobicidne mehanizme. Pomembno je predvsem, da ne zmanjšuje aktivnosti fagocitov oz. PMN periferne venske krvi, kar omogoča dvojno delovanje na tiste znotrajcelične mikroorganizme, na katere azitromicin seveda deluje. Rezultati merjenja proliferativnih odzivov s PWM in Prot A ter rezultati tvorbe tIL-2R v kulturah MNC kažejo na stimulacijo interakcije med limfociti T in limfociti B. Ponuja se hipoteza, da so morda celice pomagalke (kloni Th_2) tiste tarčne celice, na katere vpliva azitromicin.*

Zaključek. *Azitromicin vsekakor ne okvari imunskega sistema. Nasprotno, morda je imunski sistem pod njegovim vplivom celo v stanju povečane aktivnosti, kar je seveda pomembno za obrambo pred mikroorganizmi, posebno pri bolnikih z imunsko pomanjkljivostjo.*

Introduction

An interaction between antimicrobial agents and host defence factors appears to be crucial for an effective therapy of infections, especially when they are caused by the microorganisms that survive the killing mechanisms of the host phagocytes (1, 2) (fig. 1). Over the last few years, the immunomodulatory activities of antimicrobial agents have been a matter of growing concern (3). Macrolides have been extensively studied, although the results are not consistent (4). The azalide azithromycin demonstrates high volume of distribution, low levels in serum, low protein binding and a long elimination half-life (5, 6) (Tab. 1). Azithromycin is highly concentrated in a number of cell types, including polymorphonuclear leukocytes (PMN), monocytes, macrophages and fibroblasts, which can retain, deliver, and release azithromycin at sites of infection (7). The interaction between azithromycin and the bactericidal mechanisms of PMN and the macrophage system is an area of active research, but few studies have been devoted to the analysis of azithromycin interference with specific immune response (8–11).

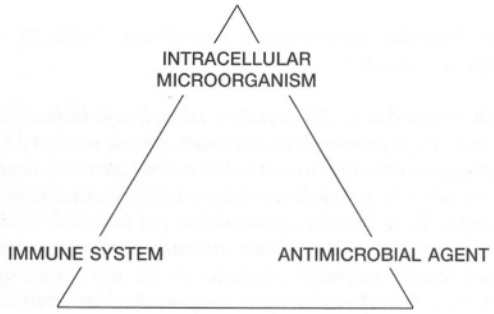

Fig. 1. *Triangle of the three equally important factors for the effective antimicrobial therapy.*

Sl. 1. *Trije faktorji, ki so enako pomembni za učinkovito zdravljenje bakterijskih okužb.*

Tab. 1. *Azithromycin pharmacokinetics in comparison with other antibiotics (modified from Schentag and Ballow 1991) (7).*

Tab. 1. *Farmakokinetične lastnosti azitromicina v primerjavi z nekaterimi drugimi antibiotiki (modificirano po Schentagu in Ballowu 1991) (7).*

Antibiotic	Cmax (mg/1)	Vd (1/kg)	t 1/2 (hours)	PMN (I/E)
azithromycin	0.4	23	14 (>40)	79
erythromycin	0.9–1.4	1.5	1.6	10–13
clarithromycin	2.4	–	4.7	–
penicillin	10.0	0.2	0.3	0.1
cefaclor	5.0	0.2	0.5	0.1
ciprofloxacin	3.5	1.4	3.9	2.5

Cmax – maximal concentration
– maksimalna koncentracija
Vd – volume of distribution
– volumen distribucije
t 1/2 – half-life time
– razpolovni čas
I/E – the ratio of intracellular to extracellular concentration
– odnos znotraj- in zunajcelične koncentracije

The aim of the present study was to investigate the effects of the therapeutic dose of orally taken azithromycin on the phagocyte microbicidal test with Candida albicans and the oxidative burst in PMN with luminol amplified chemiluminescence, its effect on the proliferative lymphocyte responses to pokeweed mitogen (PWM) and protein A (Prot A), and its influence on the amount of the soluble interleukin-2 receptors (sIL-2R) in supernatants of mononuclear cell (MNC) cultures, ex vivo stimulated by phytohemagglutinine (PHA) and phorbol myristate acetate (PMA).

The research was performed as a controlled, double blind clinical trial, comparing an azithromycin-treated group and a placebo-treated control group.

Material and methods

Azithromycin CP-62 993/XZ-450 (Pliva, Zagreb, Croatia).
The drug was administered in vivo by the oral route, 500mg daily for three days. Placebo (starch) was given in the same way.

Volunteers

The study was performed with healthy female volunteers, age 20 to 33 years. Acceptance criteria were clinical and laboratory. The selection into azithromycin (n = 21) and placebo (n = 10) group was performed as a double blind trial. Before the administration of azithromycin or placebo and seven days after that, blood samples were taken for determination of immunological parameters.

Phagocyte microbicidal test

Leukocytes were obtained from heparinized blood samples by sedimentation on dextran, followed by lysis of residual erythrocytes and by washing with Hanks' balanced salt solution (HBSS, Gibco, USA). Leukocytes were prepared in concentrations of 7×10^6/ml. C. albicans was cultured in Sabourand agar at 37°C for 24 hours followed by washing with 0.9% sodium saline, and suspended in HBSS to a concentration of 7×10^6/ml. Leukocytes and C. albicans were incubated together at 37°C for 1 hour. Sodium desoxycholat was added for lysing leukocytes. The specimens were stained with methylene blue B and the number of viable and dead C. albicans was microscopically assessed. Experiment was done in triplicate. In the control tube, the survival of C. albicans exposed to the same conditions was tested. Results were expressed as percentage (mean of three counts) of dead towards viable C. albicans.

PMN oxidative burst

PMN oxidative burst as assayed by luminol (Sigma, USA) amplified chemiluminescence method (Van Dyke). PMN from aliquots of 100 µl blood were in polyethylene vials (Clinicon, Sweden), stimulated with 250 ul 123 ng/ml phorbol myristate acetate (PMA; Sigma, USA) solution in HBSS with 1 mM sodium azide at room temperature. The solution of luminol (250 µl 1×10^{-3}M) was added, followed by measuring light emission at a wavelength of 425 nm for three minutes, using a luminometer (LKB, Wallac, Finland). Only peak values were considered. Chemiluminescence of control cells, where only luminol was added, was used for comparison, and the results were expressed in millivolts (mV).

MNC preparation

MNCs were isolated from heparinized blood by centrifugation over a Ficoll-Hypaque density gradient. The cells were then washed in RPMI 1640 (GIBCO, Scotland) supplemented with L-glutamine, streptomycine and pooled human AB serum (growth medium).

Proliferative lymphocyte responses to PWM and Prot A

MNCs were diluted with growth medium to a concentration of 1×10^6/ml. Alquots of 100 µl were placed in wells of microculture plates (NUNC, T grade, Denmark). PWM (Seromed, Germany) was added at a concentration of 10 mg/l, and Prot A (Pharmacia, Sweden) at a concentration of 12.5 mg/l, and the lymphocyte proliferative responses were measured. Control lymphocyte cul-

ture was placed in growth medium only. The cultures were incubated for 72 hours at 37°C in humidified atmosphere with 5% CO_2, after which tritiated thymidine (^{3}H-Tdr, Sorin Biomedica, Belgium) was added to each well (18.5 kBq per well) and the plates were incubated for further 18 hours. Harvesting was performed by a semiautomatic cell harvester (Skatron, Norway). After adding a scintillation cocktail, incorporation of ^{3}H-Tdr was assessed in a beta scintillation counter (LKB Pharmacia, Sweden). Results were expressed as counts per minute (CPM).

Quantitative determination of sIL-2R levels in MNC cultures

MNCs were diluted with growth medium to a concentration of 1×10^6/ml and aliquots of 1000 µl were placed in 24 wells of culture plates (NUNC, T grade, Denmark). Cells were stimulated with PHA (DIFCO, USA) at a concentration of 10 mg/l and 20 mg/l and costimulated with PMA (SIGMA, USA) in concentration of 10 µg/l. Cell cultures were incubated for 24 hours at 37°C in humidified atmosphere with 5% CO_2. Afterward the supernatant was collected with centrifugation. The quantitative determination of sIL-2R levels in the supernatant was performed by sandwich enzyme – linked immunosorbent assay with two monoclonal antibodies, which recognise two different epitopes of the sIL-2R (Eurogenetics, Belgium). Results were expressed in units per milliliter (U/ml).

Statistical analysis

The uniformity of azithromycin and placebo groups was compared before the clinical trial by the Mann-Whitney rank sum test. The significance of differences in the results before and after the clinical trial was analysed by the Wilcoxon signed rank test. Medians and quartile deviations (Q_3-$Q_{1/2}$) are presented below.

Results

Before the clinical trial there were no statistically significant differences between azithromycin and placebo groups (Tab. 2).

Tab. 2. *Medians, quartile deviations (Q_3-Q_1/2) and Mann-Whitney rank sum test between azithromycin and placebo group before the clinical trial.*

Tab. 2. *Mediane, kvartilne deviacije (Q_3-Q_1/2) in Mann-Whitneyev test vsote rangov med azitromicinsko in placebo skupino pred kliničnim preskusom.*

Test	n = 21 before azithromycin		n = 10 before placebo		Mann Whit
	median	Q_3-$Q_{1/2}$	median	Q_3-$Q_{1/2}$	p
phagocyte microbicidal	15.60	2.12	15.95	1.18	0.657
oxidative burst	4.66	1.81	6.02	1.88	0.254
stimulation with PWM	27,725	18,180	26,705	10,270	0.767
stimulation with Prot A	490	646	319	496	0.375
growth medium	283	92	249	85	0.331
level of sIL-2R	173	6.75	173	9.12	0.626

Phagocyte microbicidal test

The results are summarized in Tab. 3 A, B. Before the administration of azithromycin, the median value of C. albicans killing, expressed as a percentage of dead towards viable C. albicans, was 15.60, with quartile deviation 2.12. After ingestion of azithromycin there was a nonsignificant elevation of killing capabilities to 16.60, with quartile deviation 2.37.

PMN oxidative burst

Tables 3 A, B show the results of PMN oxidative burst assayed by chemiluminescence and expressed in mV. In azithromycin group, before the administration of the drug, the median value was 4.66, with quartile deviation 1.81. After the ingestion of azithromycin, there was a slight, but not significant elevation of median value to 4.75, with quartile deviation 2.43.

Tab. 3. *Medians, quartile deviations (Q_3-Q_1/2) and Wilcoxon signed rank test before and after the clinical trial in azithromycin group (A) and placebo group (B).*

Tab. 3. *Mediane, kvartilne deviacije (Q_3-Q_1/2) in Wilcoxonov test predznačenih rangov pred kliničnim preskusom in po njem v azitromicinski skupini (A) in placebo skupini (B).*

A – azithromycin group (n = 21)

Test	before azithromycin		after azithromycin		Wilcox
	median	Q_3-$Q_{1/2}$	median	Q_3-$Q_{1/2}$	p
phagocyte microbicidal	15.60	2.12	16.60	2.37	0.305
oxidative burst	4.66	1.81	4.75	2.43	0.821
stimulation with PWM	27,725	18,180	30,641	17,987	0.016
stimulation with Prot A	490	646	540	307	0.375
growth medium	283	92	269	70	0.931
level of sIL-2R	173	6.75	187	14.75	0.002

B – placebo group (n = 10)

Test	before placebo		after placebo		Wilcox
	median	Q_3-$Q_{1/2}$	median	Q_3-$Q_{1/2}$	p
phagocyte microbicidal	15.95	1.18	15.95	2.64	0.332
oxidative burst	6.02	1.88	5.09	1.99	0.386
stimulation with PWM	26,705	10,270	29,201	9,150	0.203
stimulation with Prot A	319	496	434	164	0.800
growth medium	249	85	235	98	0.721
level of sIL-2R	173	9.12	177	7.75	0.664

Proliferative lymphocyte response to PWM

The results are shown in Tables 3 A, B. Before the administration of azithromycin, the median value of the proliferative lymphocyte response to PWM, expressed in CPM, was 27,725, with quartile deviation 18,180. After the administration of azithromycin there was a significant (p = 0.016) elevation of median value to 30,641, with quartile deviation 17,987.

Proliferative lymphocyte response to Prot A

Tables 3 A, B show the results of lymphocyte stimulation with Prot A. In azithromycin group, before the administration of the drug, the median value of the proliferative lymphocyte response to Prot A, expressed in CPM, was 490, with quartile deviation 646. After the administration of azithromycin there was no significant change in ^{3}H-Tdr incorporation (median 540, quartile deviation 307).

Proliferative response in control nonstimulated lymphocyte cultures

The results are shown in Tables 3 A, B. Azithromycin had no significant effect on lymphocyte proliferation when lymphocytes were maintained in growth medium.

Quantitative determination of sIL-2R levels in MNC cultures

The results are shown in Tables 3 A, B. In the azithromycin group, before the ingestion of the antibiotic, the median level of sIL-2R in MNC cultures, expressed in U/ml, was 173, with quartile deviation 6.75. However, after the ingestion of azithromycin, a significant (p = 0.002) elevation in the median value was observed (median 187, quartile deviation 14.75).

Discussion

Previous studies, concerning the effect of different macrolides on cellular and humoral immune functions, have given conflicting

results (2, 3, 10, 12). The results od our study, performed on small groups, showed, that the distribution of our data obtained was not normal, Gauss. High standard deviations and wide variation ranges (unpublished data) claimed for the use of the non-parametric statistic tests. Before the beginning of the clinical trial, all variables were measured and tested, and no statistically significant differences between the two groups were found.

A global analysis of phagocyte microbicidal mechanisms was performed using phagocyte microbicidal test with C. albicans. PMN oxidative burst was assayed by the luminol amplified chemiluminescence response stimulated by PMA. We observed no significant influence of azithromycin or placebo on these functions. Our study showed that azithromycin did not impair normal phagocytic and bactericidal activity. Since normal phagocytic mechanisms in azithromycin loaded cells appeared to be intact, azithromycin may, together with natural killing mechanisms, help to eradicate the intracellular infections caused by sensitive microorganisms.

Lymphocytes have a pivotal role on the specific immune responses. It has not been established whether azithromycin accumulates in lymphocytes like erythromycin does (12).

In the present study, we examined the in vivo effect of azithromycin on human B-cell activation directly at the level of the B cells as well as at the level of the T-cell help. Prot A directly induces proliferative lymphocyte response of human B cells, while under the influence of PWM, B cells are activated via interaction with CD_4 + T cells, Th_2 clones (13). Moreover, for the production of PWM induced T-cell-derived helper factors (interleukin 4, 5, 6), cellular interactions between T cells (Th_2 clones) and monocytes (interleukin 1; IL-1) are needed (14).

Our ex vivo clinical study after the ingestion of azithromycin showed significant effect on the PWM-induced proliferative lymphocite B-cell response. However, there was no significant proliferative lymphocyte response to Prot A after the administration of azithromycin. In the control placebo group test as well as in the control laboratory test (growth medium only), there were no significant changes. Based on all the findings mentioned a hypothesis was proposed that azithromycin induced B-cell response via T-cell activation. The mechanism by which azithromycin exerted this activity was not elucidated. It remains to be determined whether this stimulation (T cells – B cells) reflects Th_2-cell activation or monocyte activation. The main target of the azithromycin effect was not established. Nevertheless, it was suggested that azithromycin might induce stimulatory activity on lymphocyte T.

This finding was supported by the results of quantitative determination of sIL-2R levels in MNC cultures, ex vivo stimulated by PHA and PMA, where the most marked effect of azithromycin on lymphocyte activation was demonstrated. The increase in sIL-2R production was statistically significant.

It has been established that after polyclonal mitogen stimulation T cells synthesize and secrete IL-2. At the same time increased expression of specific cell (T cells, B cells, monocytes) surface interleukin-2 receptors (IL-2R) are observed (15). IL-2R is composed of at least two distinct subunits, designated alpha (p55 or Tac) (16) and beta (p 70/75) (17). After MNC activation, alpha chain or Tac molecule is induced, which is expressed and released as a soluble form (sIL-2R) (18). The validity and significance of sIL-2R measurements has not yet been clarified (8–20). It has been concluded that sIL-2R is a novel marker of immune system activation, while it is directly correlated with the activation of lymphocytes and the expression of cellular IL-2R (18).

In conclusion, the overall results suggest that a therapeutic dose of azithromycin does not impair host defense system, on the contrary, it might even stimulate it. This even or potentially beneficial interaction my contribute to a successful outcome of antimicrobial chemotherapy, which means that azithromycin probably has important immunomodulatory properties, especially for immunocompromised individuals.

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References

1. Ringoir S. The "infection equation". Infection 1992; 20: Suppl 1: 75–7.
2. Labro MT, EL Benna J, Babin-Chevaye C. Comparison of the in-vitro effect of several macrolides on the oxidative burst of human neutrophils. J Antimicrob Chemother 1989; 24: 561–72.
3. Van der Auwera P. The immunomodulating effects of antibiotics. Curr Opin Infect Dis 1988; 1: 363–74.
4. Kirst HA. New macrolides: expended horizons for an old class of antibiotics. J Antimicrob Chemother 1991; 28: 787–90.
5. Foulds G, Shepard RM, Johnson RB. The pharmacokinetics of azithromycin in human serum and tissues. J Antimicrob Chemother 1990; 25 (Supplement A): 73–82.
6. Bermudes LE, Inderlied C, Young LS. Stimulation with cytokines enhances penetration of azithromycin into human macrophages. Antimicrob Agents Chemother 1991; 35: 2625–9.
7. Schentag JJ, Ballow CH. Tissue directed pharmacokinetics. Am J Med 1991; 91 (Supplement A): 3–11.
8. Gladue RP, Bright GM, Isaacson RE, Newborg MF. In vitro and in vivo uptake of azithromycin (CP – 62,993) by phagocytic cells: possible mechanism of delivery and release at sites of infection. Antimicrob Agents Chemother 1989; 33: 277–82.
9. Retsema JA, Girard AE, Girard D, Milisen WB. Relationship of high tissue concentrations of azithromycin to bactericidal activity and efficacy in vivo. J Antimicrob Chemother 1990; 25 (Supplement A): 83–9.
10. Milatovic D. Intraphagocytic activity of erythromycin, roxithromycin and azithromycin. Eur J Clin Microbiol Infect Dis 1990; 1: 35–5.
11. Bonnet M, Van der Auwera P. In vitro and in vivo intraleukocytic accumulation of azithromycin (CP-62,993) and its influence on ex vivo leukocyte chemiluminescence. Antimicrob Agents Chemother 1992; 36: 1302–9.
12. Naess A, Solberg CO. Effects of two macrolide antibiotics on human leukocyte membrane receptors and functions. Acta Path Microbiol Immunol Scand 1988; 96: 503–8.
13. Suzuki N, Sakane T, Ueda Y, Murakawa Y, Tsunematsu T. Implications for the role of cognate interactions in in vitro human B cell activation by Staphylococcus aureus Cowan I and pokeweed mitogen. J Clin Invest 1986; 77: 294–300.
14. Suzuki N, Sakane T. Mechanism of T cell – derived helper factor production upon stimulation with pokeweed mitogen in humans. Clin Exp Immunol 1989; 71: 343–9.
15. Robb RJ, Munck A, Smith KA. T cell growth factor receptors: quantitation, specificity and biological relevance. J Exp Med 1981; 154: 1455–74.
16. Leonard WJ, Depper JM, Crabtree GR et al. Molecular cloning and expression of cDNAs for the human interleukin-2 receptor. Nature 1984; 311: 626–31.
17. Teshigawara K, Wang HM, Kato K, Smith KA. Interleukin 2 high – affinity receptor expression requires two distinct binding proteins. J Exp Med 1987; 165: 223–7.
18. Rubin LA, Nelson DL. The soluble interleukin-2 receptor: biology, function and clinical application. Ann Inter Med 1990; 113: 619–27.
19. Dower SK, Smith CA, Park LS. Human cytokine receptors. Clin Immunol 1990; 10: 289–99.
20. Kroemer G, Anderu-Sanchez JL, Gonzalo JA, Gutierrez-Ramoz JC, Martinez AC. Interleukin-2 autotolerance and autoimmunity. Adv Immunol 1991; 150: 147–235.

Zdravniški vestnik

GLASILO SLOVENSKEGA ZDRAVNIŠKEGA DRUŠTVA ZDRAV VESTN, LETNIK 63, DECEMBER 1994, str. II-1–II-72, SUPPL. II

IZBRANA POGLAVJA IZ MIKROBIOLOGIJE

VSEBINA

PREDGOVOR

Izbrana poglavja iz mikrobiologije, S. Koren, V. Kotnik, M. Likar

ČLANKI

Gibanja v virologiji, M. Likar II-3

Genomske variacije in razširjenost hantavirusnih infekcij v svetu,
T. Avšič-Županc, M. Poljak II-7

Tifus - mikrobiologija in javno zdravstvo, B. Cvjetanović II-11

Interferoni pri zdravljenju tumorjev: stanje danes in izgledi za prihodnost,
W. R. Fleischmann Jr. II-15

Vloga interferonov pri uravnavanju delovanja celic in patogenezi bolezni,
R. Mažuran II-19

Odpornost tumorskih celic za interferon, C. M. Fleischmann II-23

Kronobiologija interferonskega sistema, S. Koren, W. R. Fleischmann Jr. II-27

Prašičji interferon Gama (PoIFN Gama), B. Filipič, S. Rozman, K. Carlsson, A. Cencič II-31

Tumorski nekrozni faktor - biološke značilnosti in protitumorsko delovanje,
G. Serša II-35

Produkcija rekombinantnega humanega faktorja tumorske nekroze α z dvema ekspresijskima sistemoma v bakteriji Escherichia coli,
M. Menart, V. Gaberc-Porekar, N. Kraševec, S. Miličič, R. Komel II-43

Naravna in umetna imunomodulacija,
V. Kotnik, M. Čižek-Sajko, A. Ihan, S. Simčič, B. Wraber, A. Štalc II-49

Molekule celičnih površin, ki uravnavajo ubijalsko zmožnost celic naravnih ubijalk, A. Ihan II-53

Modulacija sinteze citokinov v kulturah humanih mononuklearnih celic različnega izvora, B. Wraber II-57

Modulacija oksidativne eksplozije nevtrofilcev, S. Simčič, F. Bobanović II-63

Vpliv azitromicina na nekatere humane imunske funkcije in vivo,
J. Tomažič, V. Kotnik, S. Simčič, B. Wraber II-69