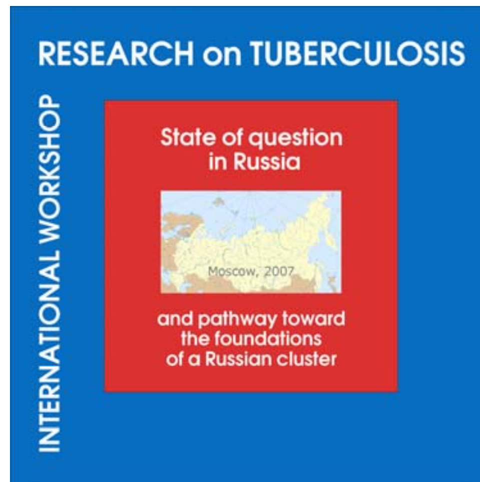

INTERNATIONAL WORKSHOP

RESEARCH ON TUBERCULOSIS
State of art in Russia
and way toward the foundation of a Russian research cluster

PROGRAMME
&
ABSTRACTS

18–19 October 2007, Moscow, Russia

FEDERAL AGENCY FOR HEALTH AND SOCIAL DEVELOPMENT
FEDERAL AGENCY FOR SCIENCE AND INNOVATIONS
RUSSIAN ACADEMY OF SCIENCES
RUSSIAN ACADEMY OF MEDICAL SCIENCES
A.N.BACH INSTITUTE OF BIOCHEMISTRY RAS
CENTRAL RESEARCH INSTITUTE FOR TUBERCULOSIS RAMS
RUSSIAN NATIONAL CONTACT CENTRE FP7 «LIFESCIENCES»

IN CO-OPERATION WITH
THE INTERNATIONAL SCIENCE AND TECHNOLOGY CENTER
RUSSIAN FOUNDATION FOR BASIC RESEARCH
AND THE EUROPEAN COMMISSION RESEARCH DG: DIRECTORATE F – HEALTH F.3:
INFECTIOUS DISEASES INTERNATIONAL COOPERATION DIRECTORATE

ORGANIZING COMMITTEE

Co-chairman – Academician (RAS and RAMS) **Vsevolod Tkachuk**,
Lomonosov Moscow State University, Russian Federation

Co-chairman – Professor **Stewart T. Cole**,
Federal Polytechnic School of Lausanne, Switzerland

Deputy head of the Federal agency for science and innovations **I. Bilenkina**, Russian Federation
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Academician (RAMS) **M. Perelman**, Russian Federation
Academician (RAS) **M. Ugryumov**, Russian Federation
Corresponding member (RAMS) **V. Erokhin**, Russian Federation
Professor **A. Apt**, Russian Federation
Professor **I. Dyatlov**, Russian Federation
Professor **A. Kaprelyants**, Russian Federation
Professor **C. Montanes**, Spain
Professor **V. Popov**, Russian Federation
Dr. **J. Thole**, The Netherlands
Partner Project Manager ISTC **N. Savinova**, Russian Federation
Project administrator of Russian Health Care Foundation **V. Grechukha**, Russian Federation

PROGRAMME COMMITTEE

Prof. **A. Apt**, Central Research Institute for Tuberculosis RAMS, Russian Federation
Prof. **S.T. Cole**, Federal Polytechnic School of Lausanne, Switzerland
Prof. **A. Kaprelyants**, Bakh Institute of Biochemistry RAS, Russian Federation
N. Savinova, Partner Project Manager ISTC, Russian Federation

SECRETARIAT

Scientific Secretary – Dr. **E. Salina**, A.N. Bach Institute of Biochemistry RAS, Russian Federation

Venue: Presidium of Russian Academy of Sciences, Leninsky prospect, 32a, President Hall

Sponsored by BIORAD

Programme at a Glance

Thursday, October 18

9.00–9.40	Registration
9.40–10.00	Workshop Opening
10.00–14.00	SESSION 1: OVERVIEW OF RESEARCH ON TB
12.00–12.15	*Coffee-break*
14.00–15.00	*Lunch*
15.15–17.15	Poster Session. Exhibition
17.30	Workshop Reception (foyer of President Hall)

Friday, October 19

9.30–11.45	SESSION 2: IMMUNOLOGICAL ASPECTS
11.45–12.00	*Coffee-break*
12.00–13.45	SESSION 3: MOLECULAR EPIDEMIOLOGY.
14.00–15.00	*Lunch*
15.00–16.30	SESSION 4. ROUND TABLE: FOUNDATION OF RUSSIAN TB CLUSTER
16.30	Closing of the Workshop

Venue: Presidium of Russian Academy of Sciences, Leninsky prospect, 32a, President Hall

PROGRAMME

18 October 2007, Thursday

9.00–9.40 REGISTRATION

Opening of the Conference

9.40–10.00 PLENARY SESSION

Vsevolod Tkachuk. Welcome speech of head of Russian National Contact Centre «Lifesciences».

Alexandr Apt. Welcome speech of Programme Committee and RFBR representative.

Waclaw Gudowski. Welcome speech of ISTC representative.

Idongesit Essiet-Gibson. Welcome speech of BTEP representative.

Richard Burger. Welcome speech of EC representative.

SESSION 1. Overview of research on TB

10.00–12.00 MEETING 1.

Co-chairmens: **Waclaw Gudowski** (ISTC) and **Alexander Apt** (Central Research Institute for Tuberculosis, RAMS)

10.00-10.20 Tatyana Gremyakova.
International Science and Technology Center, Moscow, Russia.
ISTC PROJECTS ON TB. DDD-PAT PROSPECTIVE.

10.20-10.40 Richard Burger, Hannu Laang.
European Comission, Brussels, Belgium.
THE EU SUPPORT TO TB RESEARCH.

10.40-11.00 Jelle Thole.
Division of Infectious Diseases, Animal Sciences Group, Lelystad, The Netherlands.
THE TB-VAC PROJECT.

11.00-11.20 Stewart Cole.
Federal Polytechnic School of Lausanne, Lausanne, Switzerland.
THE NM4TB PROJECT.

11.20-11.40 Aldo Tagliabue.
Novartis, Siena, Italy.
MUCOSAL VACCINES: THE HORIZONTAL APPROACH TO POVERTY RELATED INFECTIONS.

11.40-12.00 Marcus Lem.
Public Health Agency of Canada, Ottawa, Canada.
TB CONTROL IN A CANADIAN FEDERAL INSTITUTION.

12.00–12.15 COFFEE-BREAK

12.15–14.00 MEETING 2.

Co-chairmens: **Stewart Cole** (Federal Polytechnic School of Lausanne) and **Vladislav Erokhin** (Central Research Institute for Tuberculosis RAMS)

12.15-12.35 Alexander Apt.

Central Research Institute for Tuberculosis RAMS, Moscow, Russia.

IMMUNITY TO AND PATHOGENESES OF *MYCOBACTERIUM TUBERCULOSIS* – AND *MYCOBACTERIUM AVIUM* – TRIGGERED DISEASES IN MOUSE STRAINS WITH A MIRROR-TYPE GENETIC SENSITIVITY TO TWO INFECTIONS.

12.35-12.55 Vitaly Pavlov.

State Research Center for Applied Microbiology and Biotechnology, Obolensk, Moscow region, Russia.

DEVELOPMENT OF A LIVE RECOMBINANT ANTI-TUBERCULOSIS VACCINE BASED ON *F. TULARENSIS* VACCINE STRAIN.

12.55-13.15 Arseny Kaprelyants.

A.N.Bach Insitute of Biochemistry RAS, Moscow, Russia.

TB-RELATED RESEARCH AT BACH INSTITUTE OF BIOCHEMISTRY.

13.15-13.35 Olga Solopova.

Russian Research Center for molecular Diagnostics and therapy, Moscow, Russia.

SYNTHESIS AND IMMUNOCHEMICAL CHARACTERIZATION OF HUMAN ALPHA-DEFENSIN 1 AND ITS ANALOGUES IN VIEW OF ANTI-TUBERCULOSIS THERAPY DEVELOPMENT.

13.35-13.55 Sergey Nedospasov.

Engelhardt Institute of Molecular Biology RAS, Moscow, Russia.

NEW ENGINEERED MOUSE MODELS TO STUDY THE ROLE OF TUMOR NECROSIS FACTOR (TNF) IN PROTECTION AGAINST MYCOBACTERIA, IN PARTICULAR, DURING TNF BLOCKADE.

14.00–15.00 LUNCH

15.15-17.15 POSTER SESSION. EXHIBITION.

P1. Jose-Antonio Ainsa-Claver.

University of Zaragoza, Spain.

APPROACHES FOR STUDYING OF MOLECULAR BASIS OF INTRINSIC DRUG RESISTANCE IN MYCOBACTERIA.

P2. Ian Old.

Federal Polytechnic School of Lausanne, Lausanne, Switzerland.

NEW MEDICINES FOR TUBERCULOSIS (NM4TB).

P3. Sofia Samper.

University of Zaragoza, Spain.

MOLECULAR EPIDEMIOLOGY OF MULTIDRUG-RESISTANT *MYCOBACTERIUM TUBERCULOSIS* IN SPAIN (1998-2005).

P4. Sofia Samper.

University of Zaragoza, Spain.

EXTENSIVE DRUG RESISTANT (XDR-TB). A NEW NAME FOR A KNOWN PROBLEM (1998-2005).

P5. Daniela Cirillo.

Emerging Bacterial Pathogens Unit, San Raffaele Scientific Institute, Milan, Italy.

MOLECULAR DETECTION OF RIFAMPIN AND ISONIAZID MUTATIONS IN *M. TUBERCULOSIS* DIRECTLY FROM CLINICAL SPECIMENS.

P6. Paolo Miotto.

Emerging Bacterial Pathogens Unit, San Raffaele Scientific Institute, Milan, Italy.

GENOCARD: A USEFUL TOOL FOR DATA COLLECTION ON DRUG RESISTANCE AND EPIDEMIOLOGY IN TUBERCULOSIS SURVEILLANCE.

P7. Marina Janjgava.

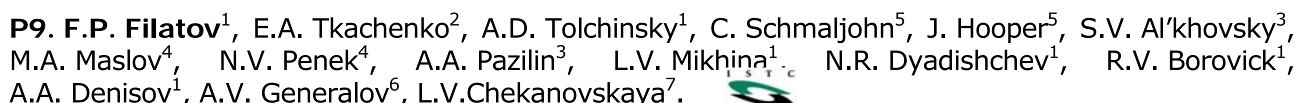
National Centre of Tuberculosis and Pulmonary Diseases, Tbilisi, Georgia.

TB CONTROL IN GEORGIA.

P8. O.I. Urazova, V.V. Novizkiy, A.K. Strelis, O.V. Voronkova, I.O. Naslednikova.

Siberian State Medical University, Tomsk, Russia.

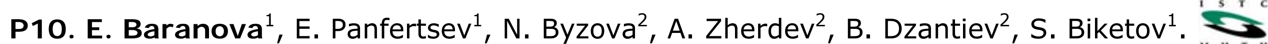
IMMUNOPATHOLOGY OF A PULMONARY TUBERCULOSIS: FUNDAMENTAL AND APPLIED ASPECTS.



P9. F.P. Filatov[1], E.A. Tkachenko[2], A.D. Tolchinsky[1], C. Schmaljohn[5], J. Hooper[5], S.V. Al'khovsky[3], M.A. Maslov[4], N.V. Penek[4], A.A. Pazilin[3], L.V. Mikhina[1], N.R. Dyadishchev[1], R.V. Borovick[1], A.A. Denisov[1], A.V. Generalov[6], L.V.Chekanovskaya[7].

[1] Research CTR for Toxicology, Serpukhov, Moscow Region, Russia.
[2] Chumakov Research Institute for poliomyelitis and viral encephalitis, RAMS, Moscow, Russia.
[3] Ivanovsky Research Institute for virology, RAMS, Moscow, Russia.
[4] Lomonosov Moscow Academy for Fine Chemical Technology, Moscow, Russia.
[5] USAMRIID, Ft. Detrick, Frederick, Medison, USA.
[6] Gabrichevsky Institute for Hygiene, Moscow, Russia.
[7] Institute for Molecular Genetics, RAS, Moscow, Russia.

DESIGN OF EXPERIMENTAL AEROSOL DNA VACCINE.



P10. E. Baranova[1], E. Panfertsev[1], N. Byzova[2], A. Zherdev[2], B. Dzantiev[2], S. Biketov[1].

[1] State Research Center for Applied Microbiology and Biotechnology, Obolensk, Moscow region, Russia.
[2] A.N.Bach Institute of Biochemistry RAS, Moscow, Russia.

REAGENTS AND TOOLS FOR RAPID TB SERODIAGNOSTICS IN RUSSIA.

P11. I.I. Lyubimov[1], S.E. Gelperina[2,3], O.O. Maksimenko[2,3], A.P. Bud'ko[2], E.V. Shipulo[2], E.V. Vanchugova[2], I.Y. Shchit[1], L.B. Heifets[4], S.F. Biketov[1].

[1] State Research Center for Applied Microbiology and Biotechnology, Obolensk, Moscow region, Russia
[2] Institute of Molecular Medicine I.M. Sechenov MMA, Moscow, Russia
[3] Research Center of Molecular Diagnostics and Therapy, Moscow, Russia
[4] National Jewish Medical and Research Center, Denver, Colorado, USA

EFFICIACY OF NANOPARTICLE-BOUND RIFAMPIN IN MICE INFECTED WITH *MYCOBACTERIUM TUBERCULOSIS.*

P12. V.I. Kiselev[1], M.A. Paltsev[1], P.M. Baranovsky[1], M.I. Perelman[2], I.V. Bocharova[3], D.T. Levy[4], A.V. Demin [5], A.M. Shuster[5], V.I. Litvinov[6], S.A. Pupshev[7].

[1] SRI of Molecular medicine PEI I.M. Sechenov MMA, ROSZDRAV, Moscow, Russia.
[2] SRI of Phthisiology and pulmonology PEI I.M. Sechenov MMA ROSZDRAV, Moscow, Russia.
[3] Central Tuberculosis Research Institute RAMS, Moscow, Russia.
[4]GISK PEI L.A. Tarasevicha, Moscow,Russia.
[5]CSC «Masterclon», Moscow,Russia.
[6]Moscow Tuberculosis Scientifically-Practical Center, [7]CSC «Veles-Farma», Russia.

RECOMBINANT PROTEIN BASED SKIN TEST FOR TB DIAGNOSTICS.

P13. O.V. Antonova, D.A. Gryadunov, S.A. Lapa, V.M. Mikhailovich, A.S. Zasedatelev, V.E. Barsky.

Engelhardt Institute of Molecular Biology RAS, Moscow, Russia.
Biochip-IMB Ltd., Moscow, Russia.

TB-BIOCHIP® DIAGNOSTIC KITS FOR FAST IDENTIFICATION OF MTB COMPLEX AND DRUG SUSCEPTIBILITY TESTING.

P14. G.S. Shepelkova, M.A. Kapina, I.V. Lyadova.

Central Research Institute for Tuberculosis RAMS, Moscow, Russia.

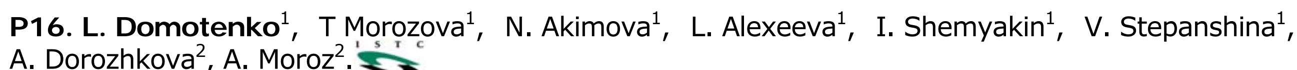
GENERATION AND MIGRATION OF $CD27^{LO}$ EFFECTOR CD4 T LYMPHOCYTES DURING MYCOBACTERIAL INFECTION IN MICE.

P15. V.A. Vavilin[1], S.I. Makarova[1], T.A. Kolpakova[2], L.A. Kozhanova[3], V.A. Krasnov[2], V.V. Lyakhovich[1].

[1] Institute of Molecular Biology and Biophysics, SB RAMS, Novosibirsk, Russia.
[2] Novosibirsk Institute of Tuberculosis of Ministery of Social Development and Health of Russian Federation, Novosibirsk, Russia.

INFORMATIVITY OF GENETIC AND PHARMACOKINETIC TESTING IN PREDICTION OF ANTITUBERCULOSIS DRUGS INDUCED HEPATOTOXICITY.

P16. L. Domotenko[1], T Morozova[1], N. Akimova[1], L. Alexeeva[1], I. Shemyakin[1], V. Stepanshina[1], A. Dorozhkova[2], A. Moroz[2].



[1] State Research Center for Applied Microbiology and Biotechnology, Obolensk, Moscow region, Russia.
[2] Moscow Science and Practical Anti-Tuberculosis Center, Moscow Government, Russia.

EVALUATION OF THE TB TEST-KIT FOR RAPID DRUG SUSCEPTIBILITY TESTING OF M.TUBERCULOSIS IN MOSCOW SCIENCE AND PRACTICAL ANTI-TUBERCULOSIS CENTER.

P17. A.A. Sheveleva, M.A. Semashko, Y.L. Dorokhov.

A.N.Belozersky Institute of Physico-Chemical Biology, Moscow State University, Russia.

SYSTEM FOR SUPEREXPRESSION OF TUBERCULOSIS VACCINE IN PLANT.

P18. A.V. Syroeshkin, T.V. Grebennikova.

Russian People Friendship University, Moscow,Russia.

APPLICATION OF MOLECULAR DIAGNOSTIC TOOLS FOR MONITORING OF TREATMENT AND TESTING OF THE NEW PHARMACEUTICAL COMPOSITION FOR ADDITIONAL CHEMOTHERAPY OF TUBERCULOSIS.

P19. Yu.S. Alyapkina[1], M.A. Vladimirsky[2], D.A. Varlamov[1], J.I. Alekseev[1], and L.K.Shipina[2].

[1] «Syntol» Ltd.Co, Russia.
[2] Research Phthisiopulmonology Institute I.M. Sechenov MMA, Moscow, Russia.

RESULTS OF *M. TUBERCULOSIS* DRUG RESISTANCE MONITORING IN RUSSIA WITH USE OF REAL-TIME PCR TECHNOLOGY.

P20. Andrey Simbirtsev.

State Research Institute of Highly Pure Biopreparations, St.Petersburg, Russia.

NEW APPROACHES TO THE TUBERCULOSIS THERAPY USING RECOMBINANT CYTOKINES AND SYNTHETIC PEPTIDES.

17.30 RECEPTION

19 October 2007. Friday

SESSION 2. Immunological aspects

9.30–11.45 MEETING 1.

Co-chairmens: **Arseny Kaprelyants** (A.N.Bach Institute of Biochemistry, RAS)
and **Jelle Thole** (Division of Infectious Diseases, Animal Sciences Group)

9.30-9.50 Roland Brosch.
Institute Pasteur, Paris, France.
TUBERCULOSIS RESEARCH AT THE INSTITUT PASTEUR – AN OVERVIEW.

9.50-10.05 Douglas Lowrie.
Cardiff University, Cardiff, United Kingdom.
THE POTENTIAL FOR IMMUNOTHERAPY.

10.05-10.25 Carlos Martin Montanes.
University of Zaragoza, Spain.
MOLECULAR SURVEILLANCE OF DRUG-RESISTANT TB AND RESERACH IN NEW LIVE VACCINE.

10.25-10.45 Georgii Kosmiadi.
Central Research Institute for Tuberculosis RAMS, Moscow, Russia.
ORGANIZED LOCAL IMMUNE RESPONSE IN THE LUNGS OF PATIENTS WITH TUBERCULOSIS.

10.45-11.05 Irina Lyadova.
Central Research Institute for Tuberculosis RAMS, Moscow, Russia.
RAPID TB PROGRESSION: ELUCIDATION OF CELLULAR AND MOLECULAR MECHANISMS AND POSSIBLE TARGETS FOR IMMUNOMODULATION.

11.05-11.25 Vladimir Lunin.
Gamaleya Institute of Epidemiology and Microbiology, RAMS, Moscow, RUSSIA.
NOVEL RECOMBINANT SUBUNIT TUBERCULOSIS VACCINES: POLYMERIC CARBOHYDRATE MICROPARTICLES AS AN ADJUVANT FOR MYCOBACTERIAL ANTIGENS FUSED WITH CARBOHYDRATE-BINDING PROTEIN DOMAIN.

11.25-11.45 Dmitry Gryadunov.
Engelhardt Institute of Molecular Biology RAS, Moscow (Russian Federation).

GEL-BASED BIOCHIPS FOR ANALYSIS OF *MYCOBACTERIUM TUBERCULOSIS*: METHODS AND APPLICATION IN CLINICAL PRACTICE.

11.45-12.00 COFFEE-BREAK

SESSION 3. Molecular epidemiology

12.00–13.45 MEETING 1.

Co-chairmens: **Marcus Lem (Public health Agency of Canada)**
and **Alexandr Ilyichev** (State Research Center of Virology
and Biotechnology «Vektor»)

12.00-12.20 Igor Shemyakin.

State Research Center for Applied Microbiology and Biotechnology, Obolensk, Moscow region, Russia.

GENETIC DIVERSITY AND POPULATION STRUCTURE OF *M.TUBERCULOSIS* STRAINS CIRCULATING IN CENTRAL RUSSIA.

12.20-12.40 Larisa Chernousova.

Central Research Institute for Tuberculosis RAMS, Moscow, Russia.

BIOLOGICAL PROPERTIES OF *M. TUBERCULOSIS* STRAINS W CLUSTER, PREVALENCE IN RUSSIA.

12.40-13.00 Sergey Tatkov.

State Research Center of Virology and Biotechnology «Vektor», Koltsovo, Novosibirsk region, Russia.

MYCOBACTERIUM TUBERCULOSIS ISOLATES FROM A RURAL POPULATION IN NOVOSIBIRSK REGION OF RUSSIA.

13.00-13.40 Olga Narvskaya.

Pasteur Research Institute of Epidemiology and Microbiology, St. Petersburg, Russia.

MOLECULAR MARKERS: APPLICATION FOR STUDIES OF *MYCOBACTERIUM TUBERCULOSIS* POPULATION IN RUSSIA.

13.40-14.00 Mikhail Vladimirsky.

Research Institute of Phtisiopulmonology Sechenov Moscow Medical Academy, Moscow, Russia.

DEVELOPMENT OF THE DIAGNOSTIC KITS BASED ON THE REAL-TIME PCR AND INTERFERON-GAMMA ANTIGEN SPECIFIC INDUCTION FOR DIAGNOSIS OF TUBERCULOSIS AND DRUG RESISTANCE OF M.TUBERCULOSIS COMPLEX.

14.00-15.00 LUNCH

SESSION 4. Foundations of Russian TB cluster

15.00–16.30 ROUND TABLE

Moderators: **Natalia Savinova** (ISTC) and **Alexander Apt** (Central Research Institute for Tuberculosis RAMS)

PANELISTS:

Alexander Ilyichev. *State Research Center of Virology and Biotechnology «Vektor», Koltsovo, Novosibirsk region, Russia.*

Maxim Filipenko. *Institute of Chemical Biology and Fundamental Medicine SB RAS, Novosibirsk, Russia.*

Ivan Dyatlov. *State Research Center for Applied Microbiology and Biotechnology, Obolensk, Moscow region, Russia.*

Arseny Kaprelyants. *A.N.Bach Insitute of Biochemistry RAS, Moscow, Russia.*

Igor Shemyakin. *State Research Center for Applied Microbiology and Biotechnology, Obolensk, Moscow region, Russia.*

Sergey Biketov. *State Research Center for Applied Microbiology and Biotechnology, Obolensk, Moscow region, Russia.*

16.30. Closing of the Workshop

A B S T R A C T S

IMMUNITY TO AND PATHOGENESES OF *MYCOBACTERIUM TUBERCULOSIS* AND *MYCOBACTERIUM AVIUM* – TRIGGERED DISEASES IN MOUSE STRAINS WITH A MIRROR-TYPE GENETIC SENSITIVITY TO TWO INFECTIONS

E. KONDRATIEVA, T. RADAEVA, V. SOSUNOV, V. EVSTIFEEV, T. KONDRATIEVA, M. AVERBAKH, **A. APT**

Central Reseach Institute for Tuberculosis, Moscow, Russia

Manifestations of mycobacterial infections can result in divergent outcomes in different individuals. The spectrum of infection with *Mycobacterium tuberculosis* ranges from rapidly progressing overt tuberculosis (TB) to asymptomatic containment. Organisms of the *Mycobacterim avium* complex are intracellular human pathogens in the absence of the normal T cell immunity. They are present in approximately 70% of patients with advanced untreated AIDS and are major killer in this cohort. On the background of less severely impaired immunity *M. avium* may cause chronic lung disease. This wide range in outcomes depends upon a complex interplay between mycobacteria and the host and is likely due to differences in the ability of genetically different hosts to develop and maintain protective *vs*. deleterious types of innate and acquired immune responses. How these responses are regulated at genetic and cellular levels remains largely unknown.

Many aspects of genetics and immunity to mycobacterial diseases in general, and TB in particular, has proven to be extremely difficult to study in humans, due to the complexity of host genetics, variations in the pathogenicity of the microbe determinants, the level of exposure, etc. Thus, a recent workshop organized by the National Heart, Blood and Lung Institute made animal models to improve understanding of persistence, reactivation, granulomatous reactions and genetic predisposition its first recommendation. We have developed a range of murine models of *M. tuberculosis* and *M. avium* infections based on natural genetic variation in inbred strains of mice, and demonstrated a mirror-type susceptibility/severity pattern of two infections in B6 and I/St inbred mice. We now use a combination of DNA microarrays, real time PCR, immunohistochemistry, and gene mapping approaches to: (i) investigate the shifts in host gene expression that accompany development of diseases caused by *M. tuberculosis* and *M. avium* infections in genetically susceptible and resistant murine hosts; (ii) evaluate the features of lung inflammation and pathology in these animals; and (iii) dissect the role of individual QTL and *MHC* genes in the control of TB infection.

We are obtaining a dynamic picture of gene expression in lungs and lymphoid organs from the onset of infection until development of contrast phenotypes in susceptible and resistant animals in order to better understand the changes in gene expression that differentiate between effective host defense mechanisms and impaired ones. Given that the two mouse strains display a mirror-type susceptibility/severity pattern of two infections, and that pathological features of two diseases are very similar, our experimental system represents a unique, self-controlling setting, which allows identification of genes whose up- or down-regulation underlies protective and deleterious types of host response. We are dissecting phenotypic differences between susceptible and resistant mice by studying the architecture of lung tissue pathology throughout the course of two infections by means of immunohistochemistry. Overall, our results suggest that: (i) mouse models of mycobacterial diseases reflect very well all features of corresponding human diseases if the choice of a mouse strain is correct; (ii) the prevalence of neutrophils in TB inflammation contributes to the development of pathology, rather than protection of the host, and neutrophils may play the role of a "Trojan horse" for mycobacteria; (iii) formation of the tertiary lymphoid follicules in the lung tissue with a prevalence of B-cells is a characteristic feature of the unfovorable course of mycobacterial infections.

To understand at the organism level the integrative phenotype "TB susceptibility" and to analyze the impact of individual QTL that we have mapped on chromosomes 3 and 9, we developed a panel of mouse strains congenic for individual QTL involved in TB control. These newly established mouse strains will be studied with respect to the TB susceptibility. If contrast phenotypes are expressed, we will define the chromosome 3 and 9 QTL map locations to approximately 1-2 cM intervals by a sequential 2-stage interval-specific congenic strains approach. Next, we plan to clone corresponding QTL relying on testing of candidate genes available from complete gene map of the mouse. If the difference at a single QTL does not provide a reliable phenotypic difference, we will produce a panel of double-congenic strains carrying all four possible combinations of chr. 3 and 9 QTL. Evaluation of TB phenotypes in double-congenic mice will define epistatic/complementary interactions between QTL which were observed in our previous studies.

BIOLOGICAL PROPERTIES OF *M. TUBERCULOSIS* STRAINS W CLUSTER, PREVALENCE IN RUSSIA

L. CHERNOUSOVA

Central Research Institute For Tuberculosis, RAMS, Moscow, Russia

The present research study has been initiated due to growing interest in *M.tuberculosis* strains W cluster. Global prevalence and frequent outbreaks of TB associated with W strains let us presume that these strains spread more actively than others. However, factors responsible for this are not comprehensively investigated.

Aim: to study genetic polymorphism and biological properties of *M. tuberculosis* strains W cluster *in vitro*, *ex vivo* and *in vivo*.

RFLP IS6110 typing of 1227 *M.tuberculosis* strains (from 9 regions of Russia, 1998 – 2003) revealed more than 42 clusters. Data showed that strains of W family (40%) and AI family (18,2%) were predominated. 19,7% of strains had non-clustered genotypes.

To describe W cluster *M. tuberculosis* polymorphism, we analyzed hybridization profiles of 413 W strains and detected 114 strain variants. Most of strains (33%) belonged to strain variant W148.

Based on the genotyping results we selected 18 sensitive clinical strains, out of which 8 belonged to W cluster and 10 – to other genetic clusters. H37Ra and H37Rv strains were used as controls.

Study of *in vitro* growth revealed the strains varied in 5,6-[^{3}H]-uracil inclusion, which indicated the level of RNA synthesis and *M. tuberculosis* replication.

Determination of mycobacterium growth *ex vivo* in macrophages after 90 hours of incubation revealed decrease of RNA synthesis in all strains versus *in vitro*. All 8 strains W cluster had high replication level versus other strains. The growth of virulent H37Rv and attenuated H37Ra in macrophages had almost 5 times difference.

Since, *ex vivo* studies involving phagocytes serve as model of early stage infection, to conclude about virulence, we carried out *in vivo* experiments, when the host's immune system is activated during development of the disease.

We used 2 mouse models: high dose infection – $5x10^6$ CFU, and, for longer observation, lower dose infection – $4x10^5$ CFU *M.tuberculosis*.

Study of TB process in mice induced by infecting with *M.tuberculosis* strains, estimated by life period, weight loss dynamics, and, for some strains, mycobacterial loading and pathomorphologic changes in the lungs, revealed that W cluster strains varied in virulence, and were either more or less virulent that H37Rv. Three *M.tuberculosis* strains: one HD cluster and two non-clustered strains, demonstrated higher virulence than W cluster strains.

In vitro, ex vivo and *in vivo* studies showed that *in vitro* W cluster strains had medium replication rate, but high viability in macrophages. *In vivo* these strains differed in virulence.

The fact that HD-cluster strains and 2 non-clustered strains were more virulent than W strains contradicts the common opinion that *M.tuberculosis* W cluster strains are hypervirulent.

However, we have detected the feature which distinguishes this cluster strains from others – that is elevated ability to survive in macrophages in any infective doses. Probably, this ability promotes successful proliferation of W cluster strains and is conditioned by peculiar expression of a number of genes responsible for interaction between the pathogen and the host.

COMPARATIVE ANALYSIS OF DIFFERENT METHODS USED FOR *MYCOBACTERIUM TUBERCULOSIS* BEIJING FAMILY STRAINS DETECTION

M. FILIPENKO[1], M. ROT[1], N. SHTEYGER[1], S. TATKOV[2], A. SIVKOV[2]

[1] *Institute of Chemical Biology and Fundamental Medicine SB RAS, Russia*
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It is known, that Beijing family *Mycobacterium tuberculosis* associates with multy drug resistance, possess heightened virulence, and often excites ignitions of disease. There are some regions of the world where Beijing family predominates. The rapid definition of isolates from this family is actual objective for phtisiatric practice, it would be helpfull for the strategy of treatment determing.

The objectives of our work are to compare different methods of determination this strains *M.tuberculosis* by specificity and sensitivity.

This work was done on collection of *M. tuberculosis* strains, composed from 76 strains which are resistant to four or more antibiotics.For detection of the *M. tuberculosis* Beijing family strains VNTR–typing of ETR–A, B, C, D, E loci have been used as referent method. The genotyping of strains was done using RD105 chromosome region, MIRU 26 locus, the IS*6110* insertion in Rv2820c.

It was shown that 43 stains (56.6%) belong to Beijing family, 21 strains (27.6%) belong to LAM family, 2 stains (2.6%) belong to Haarlem family, 10 stains (13.2%) are unique genetic variants. The diagnostic sensitivity of deligotyping by RD105 chromosome region constituted 100%, and the diagnostic specificity – 84.8%. The sensitivity of Beijing family strains detection using MIRU26 locus was 55.8%, and the specificity – 93.9%.Using real-time PCR we were able to correctly identified of 43 Beijing strains (100%). Eight non-Beijing strains (24.2%) were positive too. This number was considerably reduced to 3% after primers set correction (one positive non-Beijing sample). (The negative results were obtained only for strains of non-Beijing families (25 strains – 75.8%). Accordingly, the diagnostic sensitivity of Beijing family strains detection based on the presence of the insertion IS*6110* in Rv2820c constituted 100%.

Based on results obtained we have shown that application of only one genomic locus for detection of the Beijing family strains has limited specificity, that's why it is necessary to search for new genetic markers that might be included in the analyses.

GEL-BASED BIOCHIPS FOR ANALYSIS OF *MYCOBACTERIUM TUBERCULOSIS*: METHODS AND APPLICATION IN CLINICAL PRACTICE

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The gel-based biochips developed in Engelhardt institute of molecular biology, RAS is a powerful tool to identify specific genomic targets, mutations and single nucleotide polymorphisms. A biochip consists of semi-spherical (around 100 µm in diameter) hydrogel elements which are arranged on a hydrophobic surface at the density of 10 units per square mm. The gel elements bear highly specific probes (oligonucleotides or DNA fragments) which can interact selectively with the complementary sequences of tested DNA. To perform biochip-based analysis, the isolated, amplified and fluorescently labeled DNA/RNA is injected into a biochip microchamber where the hybridization or PCR takes place. Specific fluorescent hybridization signals are captured and and processed by a specialized biochip reader which can be used for analysis of all types of biochips produced in EIMB.

At present, a number of biochip-based diagnostic kits have been developed and manufactured for analysis of *Mycobacterium tuberculosis* complex (MTB) DNAs. The first molecular approach (TB-Biochip®) is used to identify mutations responsible for rifampin (Rif) and isoniazid (Inh) resistance of MTB strains. The mutations are located in the *rpoB*, *katG*, *inhA* genes and in the intergenic regulatory region of the *ahpC-oxyR* genes. The technique based on multiplex PCR followed by hybridization on an oligonucleotide microarray allows detection more than 95% of Rif-resistant and about 80% of Inh-resistant MTB strains in clinical samples within twenty four hours. These figures are based on analysis of > 3000 clinical samples and isolates that were tested during clinical trials performed in Russian and US laboratories. Currently, the method for fast identification of drug-resistant *M. tuberculosis* strains is approved by Russian Ministry of Health and certified to be applied in clinical practice. TB-Biochip® kit is already used in eight antituberculosis institutions in Russia, as well as in USA and Kyrgyzstan laboratories.

Another technique (TB-biochip-2®) was developed to establish fluoroquinolone (FQ)-resistance in *Mycobacterium tuberculosis* isolates and clinical samples. The method allows one to identify 8 mutant variants of DNA in FQ-resistant strains (about 85% of all resistant forms). Several hundred clinical samples and isolates were tested using the developed procedure. We found that 92% of FQ-resistant isolates had mutations in the *gyrA* gene. The naturally occurring polymorphism in codon 95 of *gyrA* gene was revealed in all resistant and 76% of susceptible strains. The sensitivity and specificity of the developed approach upon testing clinical samples were 93% and 100%, correspondingly. The TB-biochip-2® kit was also certified by Russian Ministry of Health.

In addition to microarrays analyzed the mutations responsible for drug resistance we are developing biochips to genotype MTB strains and identify species of other mycobacteria. The first biochip contains probes specific for 43 known spacers separated repeated sequences inside the direct repeat region (DRR) of mycobacterial DNA. The spoligotyping on the biochips is a useful tool for identification of genotypes belonging to Beijing family as well as for differentiation of *M.tuberculosis* from *M.bovis*. The results of genotyping could be also used for epidemiological surveillance. The biochip aimed at species identification is arranged with probes to the *gyrB* gene specific region. Rapid and acurate species identification is directed to prescribing effective antimycobacterial medication, particularly for HIV and TB co-infected patients.

TB-RELATED STUDIES AT A.N. BAKH INSTITUTE OF BIOCHEMISTRY

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1.Molecular mechanisms of latent tuberculosis (dormancy and resuscitation of *Mycobacterium tuberculosis*).

Pathogenic *Mycobacterium* spp. have evolved mechanisms to ensure their survival inside the eukaryotic host. This results in a chronic form of the disease, in which bacteria may persist *in vivo* for protracted periods without causing apparent disease. These bacilli presumably pass into a latent (dormant) state during which growth is either very slow or non-existent.

We developed a model in which *My.tuberculosis* cells grown under sub-optimal conditions and prolonged incubation in stationary phase bacteria adopted a stable "non-culturable"(NC), dormant state. The viability of NC cells could be restored (resuscitated) by incubation of cells in liquid medium supplemented by supernatant taken from growing cultures. Secreted proteins similar to Rpf (resuscitation promoting factor secreted by *Micrococcus luteus*) appeared to be responsible for the activity in the culture supernatant. Rpf homologues have been found in a number of GC-rich Gram-positive bacteria, including *M. tuberculosis* (5 genes) and *M. smegmatis*(4 genes). Mutants of *M.tuberculosis* with deleted *rpf* genes (triple mutations) were unable for resuscitation *in vitro* and showed significant attenuation of their virulence *in vivo*. These strains were also defective in their ability to re-grow after imunosupression in a chronic mouse model. We suggest that one (or more) of the cognate *My. tuberculosis* Rpf–like molecule(s), could be involved in mechanisms of latency and reactivation of tuberculosis *in vivo*.

At the same time attenuated strains of *M.tuberculosis* could be used as live vaccines (the corresponding experiments are in progress).

Recent solution of Rpf structure suggests the presence of "lysozyme fold" in its molecule. We found muralytic activity of Rpf protein which opens a new interesting avenue to design low molecular weight compounds against Rpf function which could serve as potent inhibitors of reactivation of latent tuberculosis. The results of study of newly discovered inhibitors against Rpf enzymatic activity and resuscitation of mycobacterial cells *in vitro* will be presented.

2. Microspherical particles with rifampin for design of anti-tuberculosis drugs with prolonged mode of action.

The PHB (poly(3-hydroxybutyrate)) micro particles impart to the drug delivery systems the new characteristics, namely rate control bioresorption and high biocompatibility. Original biotechnology of PHB production elaborated at Bakh Institute enables to design the PHB with tailor-made molecular weight and controlled content of monomeric units in PHB copolymers.

In particular, PHB microspheres and nanospheres loaded with rifampicin were obtained.

It was shown that mechanism of drug release from microspheres includes both diffusion in polymer matrix and biodegradation of the PHB simultaneously.

The fixation of pharmacologically active component with the PHB and following slow drug release from the microparticles provides an optimal level of drug concentration in local target organ during long-term period that provides effective pharmaceutical action.

3. Accelerated detection of MTB cells for diagnostics of tuberculosis.

see abstract "Accelerated detection of MTB cells for diagnostics of tuberculosis based on resuscitation of dormant cells and their immune detection" by Shleeva et al.

ORGANIZED LOCAL IMMUNE RESPONSE IN THE LUNGS OF PATIENTS WITH TUBERCULOSIS

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The mechanisms of effector immunity and immunologic correlates in tuberculosis are largely unknown despite a large range of research in this field. Apparantely, studies on the development of local immune response to *Mycobacterium tuberculosis* could cast some light onto these mechanisms. This work was aimed at studying morphological structure and functions of immune cell infiltrates in face of chronic mycobacterial infection in humans. Phenotype and certain biological functions were studied such as proliferative and cytokine production responses, antigen presentation and antibody production in vivo and in vitro in response to antigen stimulation.

Arrangement of cellular infiltrates in the infected lung tissue sections was assessed using monoclonal antibodies. Mycobacterial load on the lung and quantities of infiltrating inflammatory cells were studied, as well as ratio of macrophages to lymphocytes was counted in cytospin slides. We show that infiltrating lymphocytes of different phenotypes (CD3, CD4, CD8, CD16, CD19) as well as macrophages and antigen presenting cells place themselves not in a chaotical, but rather organized manner within the granulomatous tissue. They bear markers of activation and differentiation and are capable of proliferation, cytokine production and synthesis of specific IgG, IgA and IgM antibodies in response to stimulation with mycobacterial antigens in vitro. Lymphoid follicle-like structures in the lung tissue were associated with the diagnosis of tuberculoma, but not active cavitary TB, which indicates an active role for these follicle-like structures as a prerequisite for an effective local protection against mycobacterial spread in patients with diagnosed TB and, possibly, in latent TB infection. The results suggest that chronic mycobacteria induced inflammation is associated with organization of immune cells in a morphologically discernable follicle-like organ bearing features of a tertiary lymphoid organ analogous to that found in autoimminity-affected tissue. We propose that these tertiary lymphoid organs orchestrate the local immune response to mycobacteria in humans.

NOVEL RECOMBINANT SUBUNIT TUBERCULOSIS VACCINES: POLYMERIC CARBOHYDRATE MICROPARTICLES AS AN ADJUVANT FOR MYCOBACTERIAL ANTIGENS FUSED WITH CARBOHYDRATE-BINDING PROTEIN DOMAIN

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An urgent need for the development of innovative TB vaccines is defined by imperfection of BCG, the only currently available vaccine against TB. BCG failings include, almost certainly, inability of this vaccine to protect against the most prevalent form of TB - pulmonary tuberculosis in adults, interference with skin testing, variable performance in different regions of the world, low efficacy in tropical regions endemic for mycobacterial species and obstacles for administration to HIV-positive individuals. One of the most promising approaches to develop novel TB vaccines is an elaboration of subunit vaccines consisting of either one or a few secreted proteins/peptides from *Micobacterium tuberculosis* mixed with an adjuvant. Presently the main limitation to a rapid clinical application of subunit vaccines is obvious: highly reactogenic and thus hazardous adjuvants are required to reach appreciable immunogenicity and protectivity. The aim of this project is to develop a new recombinant subunit TB vaccine candidates based on the new adjuvant technology, and to test the efficacy and safety of these novel vaccines in an animal TB model.

To achieve this goal, we intend to use a biotechnological approach established in lab of Dr. Lunin during preliminary studies, which includes construction of fusion proteins, composed from two components: (i) mycobacterial antigen(s) and (ii) polymeric carbohydrate binding domain (CBD). CBDs provide an one-stage immobilization of CBD-carrying fusion proteins on the polymeric carbohydrate microparticles. The immobilization of mycobacterial antigens on the carbohydrate carrier results in stabilization and retaining of their antigenic properties for a long time – a feature important for vaccine standardization and storage. A low cost of carbohydrate sorbents and a unique option to use such sorbents for both antigen purification and immobilization should result in an essential decrease of manufacture cost. The capacity of carbohydrate microparticles to serve the adjuvant component of a subunit TB vaccine will be tested with a set of recombinant proteins from *M. tuberculosis* with an established record of protectivity in mixtures with conventional adjuvants: Ag85A, MPT64, ESAT-6 and Cfp10. In addition, we intend to select and further test artificial antigens consisting of 2–3 immunodominant epitopes from the most protective vaccine candidates fused into a single product with a CBD and immobilized on cellulose microparticles. All novel candidate vaccines developed within the frames of this proposal will be tested in a well-established mouse model in the lab of Dr. Alexander Apt (Central Institute for Tuberculosis, Moscow, Russia). Protective effect in mice vaccinated with mono- and oligo-component experimental carbohydrate-bounded vaccines will be compared to that in animals vaccinated with identical antigens in IFA and/or MPL/DDA adjuvants, as well as with BCG (golden standard). Sham-vaccinated (adjuvant alone) mice will serve negative controls. CFU counts in lungs and spleens, as well as mean survival time, will be compared between experimental groups. To assess whether or not carbohydrate-based adjuvant is less reactogenic and more safe than traditional adjuvants, gross pathology and histological changes in skin and lymph nodes draining the site of inoculum will be examined. Once particular vaccine preparation(s) is proved to be highly protective and safe, key immunological parameters (e.g., IFN-γ production, macrophage effector function), in response to vaccination will be evaluated in corresponding group(s) of mice and compared with control groups.

RAPID TB PROGRESSION: ELUCIDATION OF CELLULAR AND MOLECULAR MECHANISMS AND POSSIBLE TARGETS FOR IMMUNOMODULATION

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In different individuals infection with *M. tuberculosis* results in divergent outcomes which range from asymptomatic latent infection to rapidly progressing disseminated disease. The exact mechanisms which distinguish protective *versus* pathological reactivity of the host and determine establishment of latency, development of active TB, and disease progression remain unclear. To elucidate these mechanisms we have developed a new experimental approach based on the analysis of immune responses in genetically heterogeneous (A/Sn x I/St)F2 mice originating from TB-resistant (A/Sn) and TB-susceptible (I/St) strains and segregating with regard to TB susceptibility. The major phenotype associated with rapid TB progression was exacerbated expression of IL-1β, IL-6, TNF-α, MIP-1α, MIP-1β, and MIP-2 inflammatory factors in the lungs. The excessive expression of inflammatory factors in mice with rapidly progressing TB was associated with lung phagocytes, but was not due to their "generalized" over-activation, as expression of "effector" factors (e.g. iNOS), was not increased. Excessive expression of inflammatory factors by lung phagocytes did not depend on mycobacterial load, but rather was due to genetically determined peculiarities of phagocyte reactivity to *M. tuberculosis*. Besides exacerbated inflammatory response, mice with rapidly progressive TB were characterized by increased expression of the immunoregulatory cytokine IL-11, and a decrease in the frequency of fully differentiated $CD27^{low}$ CD4 T cells in the lungs. Both, IL-11 and $CD27^{low}$ CD4 T cells seem to be involved in the TB control. Analysis of the mechanisms which mediate their participation in the TB control, suggests that IL-11 contributes to TB progression by promoting proinflammatory activity of lung phagocytes. $CD27^{low}$ CD4 T cells participate in TB protection: their generation dampens inflammatory and augments effector phagocyte responses. Thus, rapid TB progression is associated with uncontrolled inflammatory response of lung phagocytes, which is determined genetically, and depends on the levels of local expression of IL-11 and the degree of CD4 T cell differentiation. The possibility to use inflammatory cytokines and CD27 as potential targets for TB immunomodulation is under investigation.

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MOLECULAR MARKERS: APPLICATION FOR STUDIES OF *MYCOBACTERIUM TUBERCULOSIS* POPULATION IN RUSSIA

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Molecular markers proposed to date for characterization of *Mycobacterium tuberculosis* (MBT) complex populations allow to achieve different levels of interstrain discrimination for the aims of diagnostics, epidemiological investigations and study of *M. tuberculosis* phylogeny. Strain variability within *M. tuberculosis* complex generated by polymorphism of the chromosomal DR locus is detected by spacer oligonucleotide typing (spoligotyping). Insertion sequence IS*6110* is exploited for restriction fragment length polymorphism (RFLP) analysis of the whole genome. Mycobacterial interspersed repetitive units (MIRU) typing is based on size analysis of the PCR-amplified variable number of tandem repeats (VNTR) loci. Each method yields a DNA profile which is specific for the examined strain. Combination of the above methods provides sufficient discriminatory ability and information on epidemiological and phylogenetic links between the strains and thus is successfully used in population based studies and analysis of tuberculosis outbreaks. Mutations in *rpoB* (RIF resistance), *katG315* (INH), *embB306* (EMB), *rpsL43* (STR) can be detected in isolates and/or directly from glass slides by PCR based (allele-specific PCR and/or RCR-RFLP assays) and reverse hybridization macroarray techniques.

Using such approach, we characterized more than 700 *M. tuberculosis* clinical strains isolated since 1996 from newly diagnosed and previously treated patients in northwestern Russia including the megalopolis of St. Petersburg. The presence of several genetic families (Haarlem, Latin-American-Mediterranean etc.) and a high proportion (about 50%) of the Beijing family genotype have been identified. The Beijing strains are generally believed to cause more severe tuberculosis which was observed also in a studied population. The Beijing family genotype was first identified in China in 1992 and it is now geographically omnipresent with supposed endemicity in East Asia and the former USSR. These genetically closely related strains demonstrate some important pathogenic features such as, high transmissibility, increased virulence in BCG-vaccinated mice, association with multdrug-resistance, ability to more rapidly multiply in human macrophages and presumably easier adaptation to the changing environment due to mutator alleles of the *mutT* genes. A diversity of RFLP patterns was shown among Beijing genotype strains. However, a high proportion of clustering among unlinked Beijing strains was observed suggesting their recent transmission in our setting. In one instance, a nosocomial outbreak was caused by MDR Beijing strain as revealed by molecular epidemiological investigation. Mutations in three *rpoB* codons (516, 526, 531) were detected in 86% of RIF-resistant strains. *katG* S315T mutation was detected in 93.6% of INH-resistant strains. A mutation in *embB306* was found in 48% EMB-resistant and, surprisingly, in 31.2% EMB-susceptible strains. A mutation in *rpsL43* was detected in 44% STR-resistant strains.

NEW ENGINEERED MOUSE MODELS TO STUDY THE ROLE OF TUMOR NECROSIS FACTOR (TNF) IN PROTECTION AGAINST MYCOBACTERIA, IN PARTICULAR, DURING TNF BLOCKADE

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TNF is one of host defense factors critical for protection against intracellular bacteria and for granuloma formation. Considering that over 1 million patients worldwide with autoimmune diseases are treated by continuous blockade of TNF signaling, we want to understand the immunological consequences of TNF ablation in vivo, as it occurs in these patients. We used two approaches to generate novel and potentially useful animal models. First, we generated a panel of mice with conditional inactivation of TNF, either in specific types of leukocytes or with inducible genetic TNF ablation (Mx-Cre-TNF mice). In the latter case TNF gene deletion occurs with high efficiency in hematopoietic cells, yet these mice retain some residual TNF signaling, similarly to the patients on anti-TNF therapy. We demonstrate that such blockade disrupts some, but not all TNF-mediated functions. Secondly, we have engineered a complex transgenic/knockout mouse bearing the genomic segment encompassing the human TNF/LT locus, lacking murine TNF and LT genes, and thus being effectively "humanized" for TNF and LT. In these mice human TNF has fully compensated the absence of murine TNF in several pathophysiological models, including protection against TB. From our animal studies it appears unlikely that the beneficial effects of anti-TNF therapy will not be accompanied by intrinsic "side effects" due the loss of other TNF-mediated beneficial functions. However, these models may help to establish relative thresholds for distinct TNF functions and to define optimal blockade regiments.

DEVELOPMENT OF A LIVE RECOMBINANT ANTI-TUBERCULOSIS VACCINE BASED ON *F. TULARENSIS* VACCINE STRAIN

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Tuberculosis is a major health emergency, particularly in association of this problem with multidrug resistance and human immunodeficiency virus infection. The current vaccine against tuberculosis, bacille Calmette-Guerin (BCG), can prevent tuberculosis in young children, but the protection that BCG confers against the pulmonary tuberculosis, is not satisfactory. As the majority of the world's population is either latently infected with *Mycobacterium tuberculosis*, sensitized by exposure to mycobacteria from the environment or already BCG-vaccinated, an new vaccine should be efficient if administered on top of this exposure. The vaccine used for a booster vaccine therefore needs to be carefully selected and live mycobacterial vaccines (such as recombinant BCG) are not proper for this purpose.
Live, replicating, attenuated strains of intracellular microbes may be used as base for development of vaccine with long-lasting immunity.

The problem of choice of bacterial vectors for recombinant vaccine development is now widely discussed in the literature. Vaccine BCG and attenuated strains of Salmonellae are most frequently applied as carriers of heterologous protective antigens.

Anti-tularemia vaccine strain 15 are avirulent for human and exhibit a reduced virulence for small-sized rodents. It was developed in USSR in the middle of XXth century. Tularemia vaccine induces an intensive reorganization of the immune system and generates a long-term immunity. Process of immunization with tularemia vaccine is accompanied induction of interferon gamma and interleukin-12. These cytokines are important for induction of cell immunity.

This work is aimed at developing of recombinant *F. tularensis* strains carrying foreign protective antigens on the base of *F. tularensis* vaccine strain 15/10.

M. tuberculosis protective antigens: SOD A, ESAT6 and 85B were chosen as perspective antigens. Plasmid vector pPMC1 were constructed for *M. tuberculosis* genes expression. Replicon pFNL10 was used as base of pPMC1.

Structural parts of genes encoding *M. tuberculosis* protective antigens were inserted in plasmid vector pPMC1 as a part of a operon under *F. tularensis* GroE promoter. Western blot with anti-SOD A, ESAT6 and 85B sera was used to demonstrate of *M. tuberculosis* genes expression in *F. tularensis*. It was demonstrated that recombinant genes consisting leader part of *F. tularensis fop A* and structure part of *sod A* or *85B* genes were expressed in *F. tularensis* more effective than genes without *fop A* leader part.

It was developed molecular tool for gene insertion in *F. tularensis* 15/10 chromosome. This tool could be used for creation of *F. tularensis* strain with chromosomal localization of *M. tuberculosis* gene.

T-antigenic determinants of antigens ESAT 6 and 85B were inserted in *M. tuberculosis* SOD A protein and were expressed in *F. tularensis* 15/10.

Growth characteristics of recombinant strains in liquid media, macrophage-like cells and experimental animals were same as one of recipient strain 15/10. High level of synthesis of an interferon gamma was observed in immune mice splenocytes under *M. tuberculosis* SOD A, 85B and ESAT 6 antigens activation, than in native splenocytes. Mice vaccinated with *F. tularensis* recombinant strains caring of *M. tuberculosis* SOD A, 85B and ESAT6 antigens were protected in a some degree from aerosol challenge with *M. tuberculosis* strains H37Rv and Erdman.

GENETIC DIVERSITY AND POPULATION STRUCTURE OF *M.TUBERCULOSIS* STRAINS CIRCULATING IN CENTRAL RUSSIA

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We studied genetic diversity and drug susceptibility pattern of *M. tuberculosis* strains recovered from culture-positive pulmonary TB patients in Central Russia.

Methods. Genetic diversity was analyzed using a sample of epidemiologically unlinked strains of *M. tuberculosis* recovered in the Moscow, Tula, and Kaluga regions in 1998-2006. For population-based study all culture-positive TB patients diagnosed in Tula TB prophylactic centre and all patients of Ozerki prison hospital (Tula region) during 2001-2002 were enrolled. Drug susceptibility testing for isoniazid, rifampicin, ethambutol, streptomycin, and kanamycin was performed using method of absolute concentrations. Epidemiological markers, such as a number of tandem repeats in DR locus, IS*6110*-RFLP pattern, and MIRU-VNTR typing along with phylogenetic characteristics were analyzed. Prevalence of mutations conferring drug resistance was examined.

Results. Analysis of *M. tuberculosis* phylogenetic groups prevalent in the region revealed moderate diversity: members of four from seven SNP clustered group were found. One hundred and five different IS*6110*-RFLP patterns, 72 different spoligotypes, and 56 unique MIRU-VNTR patters were identified within the sample. Genetic distance was calculated using Jaccard’s distance matrix and corresponding phylogenetic trees were built. Members of ancient SCG2/PGG1 and modern SCG5/PGG2 showed relatively short genetic distances within their group. Members of SCG3/PGG2 and SCG6/PGG3 groups were less common and showed more divergent genotypes.

Study IS*6110*-associated polymorphisms in *plcABC* locus of *M. tuberculosis* revealed that majority of SCG5/PGG2 were clonal variants of the strain we designated as LAM-RUS. This strain is characterized by IS*6110* insertion into a unique position in *plcA* gene.

Comparative analysis of population structure of *M. tuberculosis* strains recovered in Tula TB prophylactic centre and Ozerki prison hospital during 2001-2002 showed high rate of clustered cases in both settings. Largest clusters were identified in LAM-RUS and Beijing families. Although major *M. tuberculosis* strains circulating in the sentinel and civilian population was essentially the same, higher overall level of clustering and rate of MDR TB among the new patients were found in the prison hospital. Clusters comprised from isolates recovered in both prison and civilian hospitals, emphasizing the interdependence of two populations.

High rate of drug resistant strains was observed in the region: less than 20% cases in the civilian population and about 9% cases in penitentiary were sensitive to all drugs tested. Thirty five % cases in Tula TB centre patients and 71% cases in prison hospital were MDR TB. 100% of MDR cases in the prison hospital and in civilian setting were due to a limited number of *M. tuberculosis* strains, belonging to LAM-RUS and Beijing family. Abundance of MDR strains and MDR strains resistant to kanamycin circulating in the region are of major concern implying the threat of XDR TB epidemics.

Conclusion. *M. tuberculosis* Strains LAM-RUS and Beijing families are major contributors to the TB epidemiological picture of the studied population.

SYNTHESIS AND IMMUNOCHEMICAL CHARACTERIZATION OF HUMAN ALPHA-DEFENSIN 1 AND ITS ANALOGUES IN VIEW OF ANTI-TUBERCULOSIS THERAPY DEVELOPMENT

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Anti-tuberculosis (TB) multiple drug resistance (MDR) is a major public health problem that threatens the success of DOTS, the WHO-recommended treatment strategy for detection and cure of TB, as well as global tuberculosis control. Thus the search for novel effective drug is becoming of vital importance.

The human α.-defensins (HD1,-2, and-3) are natural antimicrobial peptides. Although studies of the immunomodulatory properties of these peptides are in their infancy, there is a growing body of evidence suggesting that the immunomodulatory properties of these small, naturally occurring molecules might be harnessed for development as novel therapeutic agents. Today’s implication of defensins is limited by two main disadvantages: first, there are no convenient and inexpensive methods of defensins production and, second, the occurrence of side effects of mainly pro-inflammatory action.

Human alpha-defensins are difficult to be chemically synthesised due to their cyclic structure and three S-S bonds. Small size and toxicity for bacteria and fungi complicate the recombinant protein approach. We produced recombinant proteins by fusing HD 1-3 with mycobacterial HSP70. This construction reveals no toxicity for host E.coli and contains enterokinase site between HD and HSP70.

Pro-inflammatory activity of alpha-defensins seems to be mediated through any receptor interaction. In our research we are in the search of the defensins homologues that could not bind with defensin receptor without the loss of antimicrobial activity and moreover gaining better anti-tuberculosis properties.

MYCOBACTERIUM TUBERCULOSIS ISOLATES FROM A RURAL POPULATION IN NOVOSIBIRSK REGION OF RUSSIA

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In the last decade the tuberculosis again became the global problem for the world health system. The incidence of tuberculosis (TB) in Novosibirsk region has also increased significantly upto 137 TB cases per 100 000 inhabitants in 2003. The increasing of multidrug resistant (MDR) TB cases is going on.

For characterizing MDR strains of *M.tuberculosis*, circulating in Novosibirsk region, the representative collection of them was formed and characterized by biological methods; and epidemic, demographic data, associated with it, was sampled.

In the Novosibirsk region TB cases numbers depend on the primary patient social status, their inhabitancy and vary from 46 to 236 per 100 000 inhabitants.

The outbreaks of the sensitive TB, that we have revealed, do not match those for MDR TB in the Novosibirsk Region.

The proportion of MDR TB cases in the Novosibirsk Region is higher than the official data and keeps growing. In the MDR outbreaks the MDR proportion has varied from 5-10% to 25-30%.

The key roles in increasing MDR TB proportion in the Novosibirsk Region should be assigned to two factors working together: an ongoing selection of drug-resistant strains in specialized anti-tuberculosis hospitals followed by spread of them among the most sensitive people.

The population of *M. tuberculosis* strains in the Novosibirsk Region is genetically diverse. The most widespread isolates belonged to the Beijing strain family.

There is the correlation between the RFLP and MIRU genotyping results of a representative sample of *M. tuberculosis* strains, obtained in 2003 from patients newly diagnosed for tuberculosis; however, each cluster, detected by either technique, is a necessary but not sufficient condition for postulating epidemic links between clustered patients. When isolates of the representative sample were classified by the combined RFLP-MIRU typing the numbers of clusters were decreased and each cluster contained less number of isolates.

A search was made for mutations associated with resistance to rifampicin and (or) isoniazid. The Ser531Leu mutation has the highest proportion (74.3 %) among the rifampicin-resistant *M. tuberculosis* strains. The most frequent mutation in the isoniazid-resistant *M. tuberculosis* strains obtained from the patients in the West Siberian region is Ser315Thr of the *Kat*G gene (91%). Therefore, this mutation detecting can be recommended for the rapid testing of isoniazid resistance.

The resistance to rifampicin is the reliable marker for the MDR strain which are resistant both to rifampicin and isoniazid in Novosibirsk region, as not less than 94% of the rifampicin-resistant isolates have been resistant to isoniazid too.

EVELOPMENT OF THE DIAGNOSTIC KITS BASED ON THE REAL-TIME PCR AND INTERFERON-GAMMA ANTIGEN SPECIFIC INDUCTION FOR DIAGNOSIS OF TUBERCULOSIS AND DRUG RESISTANCE OF *M. TUBERCULOSIS COMPLEX*

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We developed diagnostic test systems utilizing real-time PCR technology (rtPCR) for quantitative determination of tuberculosis complex mycobacteria (MTB) in clinical samples and detecting major mutations associated with resistance to rifampicin, isoniazid and etambutol. Detecting mutations in rpoB (codons 511, 516, 526, 531 and 533), katG 315 and inhA-209 genes, as well as in embB 306 gene were performed using a modified allele-specific rtPCR. The method is based on using linear fluorogenic DNA probe (Taq Man) for detecting MTBDNA along with a system of 3 specially constructed allele-specific primers 5'-labeled with different fluorescent markers complementary to oligonucleotides with a fluorescence quencher at 3'-terminus. In the absence of mutation, only non-labeled primers and linear fluorogenic TaqMan-probe produce the fluorescent response. When mutation is present, allele-specific primer is incorporated in a gene fragment tested, which is accompanied by release of a quencher resulting in an increase of fluorescence from the respective fluorescent moiety. Thus up to 3 point mutations can be detected in one tube.This method was used to analyze about two thousand MTB strains, 70% of which were from newly diagnosed treatment-naïve patients from 24 areas from main geographic zones of Russian Federation. The method thus permitted to demonstrate the prevalence of resistance to rifampicin and isoniazid in Russia. In average, primary and secondary multidrug resistance (MDR) occurred in 21.9% and 58.5% of patients, respectively. By comparing our data with the results of microbiological examination of 1092 strains analyzed in 11 regional laboratories by means of absolute concentrations method, the concordance of both methods was demonstrated (with an average concordance rate of 94%). Retesting of prospective sampling from 100 cultures by means of proportion method (in the Institute of Applied Microbiology, Obolensk) showed the concordance rate of 99,5%.

To detect the corresponding mutations in sputum samples, standard quantitative PCR-reaction was performed, and the number of IS6110 copies determined. If $\geq 10^4$ IS6110 copies (about 10^3 genomes) were present, mutational analysis was conducted with respect to mutations associated with drug resistance in rpoB, katG, inhA. For this purpose, DNA fragments from the relevant genes were amplified using a preliminary short (20 cycles) round of multiplex PCR. After that, mutations were analyzed using fluorescently labeled primers as described above. In general, the results of sputum testing were concordant with those of testing resistance to rifampicin and isoniazid in the same samples using Bactec system, the concordance rate being 93.6%. Furthermore, number of interpretable results of sputum samples analyses was higher for rtPCR method than for that based on Bactec system. Recently, the same technology was successfully applied to detect mutations of gyrA gene (codons 90, 94) associated with resistance to ofloxacin.

We also developed kit for diagnosis of tuberculosis infection in children and teenagers. Despite BCG immunization of new-born children followed by two reimmunizations, which are the mandatory in Russia, tuberculin skin-test often can not be used for detection of tuberculosis infection and its differential diagnosis from postvaccinal allergy. We have developed and tested diagnostic system based on induction of interferon-gamma in the whole blood of pediatric patients in the presence of two antigens: PPD tuberculin and specific recombinant antigen ESAT-6. Interferon-gamma is determined be means of enzyme-linked immunosorbent assay utilizing 2 monoclonal antibodies against two different epitopes of interferon-gamma. By using this method, we have shown the high level of response to both antigens in all patients (n=85) with primary tuberculosis infection. In 90% of samples from patients with postvaccinal allergy, only response to PPD tuberculin was induced with no response to specific ESAT-6 antigen. We also used this method to assess the level of immune response in teenager patients with local forms of pulmonary tuberculosis. In those with severe lesions, the response to both antigens was rather low, but it restored with efficient treatment. On the other hand, in patients with initially high levels of interferon-gamma induction, we observed its decrease in the course of specific chemotherapy. These studies may become the basis for thorough and adequately controlled use of immunotherapeutic regimens.

POSTERS

NEW MEDICINES FOR TUBERCULOSIS (NM4TB)

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Summary

New Medicines for Tuberculosis (NM4TB) aims to successfully develop new drugs for the treatment of tuberculosis (TB) through an integrated approach implemented by a team that combines some of Europe's leading academic TB researchers with a major pharmaceutical company and three SMEs, all with a strong commitment to discovering new anti-infective agents. NM4TB has a comprehensive portfolio of potential and validated targets plus several novel, proprietary anti-TB agents in its drug development pipeline. Among the validated targets are several enzymes involved in highly druggable areas such as cell wall biogenesis, nucleic acid synthesis and central metabolic pathways for which assays amenable to high-throughput screening are available. Intensive efforts will focus on rapidly emerging targets that impact upon two as-yet untouched areas of the physiology of Mycobacterium tuberculosis signal transduction pathways and persistence.

Background

Tuberculosis (TB) is one of the oldest diseases known to man and has infected one third of the world's population. As a result, someone dies from the disease every 15 seconds and 30 million more people will lose their lives to TB in the next decade. Although directly observed short course chemotherapy is available to treat the disease, this treatment is old, slow and inefficient by the current standards of the pharmaceutical industry. Here, we will employ the most innovative approaches to identify and validate targets for new drugs, and implement the screening and medicinal chemistry processes required to identify lead compounds for the generation of candidate drugs.

Aim

To successfully develop new drugs for the treatment of tuberculosis (TB) with the following desired properties:

1) High potency to reduce treatment duration,

2) Activity against persistent bacilli,

3) Inhibition of new target classes,

4) Activity against multidrug resistant TB,

5) Specificity for M. tuberculosis.

Expected results

1) Development and implementation of novel enabling technologies required for drug development.

2) Target validation in well-established, "druggable" areas such as the central metabolism, cell wall and nucleic acid synthesis in addition to more challenging yet highly innovative topics like the signal transduction and persistence mechanisms.

3) Generation of the structural information for as many targets as possible, acting iteratively in the drug development process. Structures of targets with drugs bound to rationally improve drug design.

4) Assay development and screening of deep chemical libraries encompassing "Active" to "Hit", "Hit" to "Lead" progression, "Lead" optimization activities that give rise to candidate drugs.

Potential applications

The proposed research will result in:

1) Development of new technologies and assays for TB drug development;

2) Discovery of new classes of lead compounds for fighting TB;

3) Lead optimization and progression to candidate drug status.

Keywords

Mycobacterium tuberculosis, multidrug resistant TB, drug development, signal transduction pathways.

RESULTS OF *M. TUBERCULOSIS* DRUG RESISTANCE MONITORING IN RUSSIA WITH USE OF REAL-TIME PCR TECHNOLOGY

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Spread of drug-resistant strains of MTB is one of the most important problems in the world as well as in Russia. At present there are contradictory data on the spread of MTB strains with multiple drug resistance in Russia.

The modified technique of allele-specific real-time PCR for detection of MTB DNA mutations was used. We developed a one-tube assay to use simultaneously a set of 5'-fluorescent allele-specific primers and a complementary to the common part of the primers oligonucleotide labeled with a quencher at 3'-end and the control TaqMan probe complementary to the central conservative part of the fragment. If there is no mutation in the DNA of sample the standard real-time PCR will take place and only the fluorescence of the TaqMan probe fluorophore will increase. If a sample contains mutated DNA the fluorescence increment of both the TaqMan probe and the allele-specific primer will be registered. This system allows to determine not only the mutation but the percentage of the mutant and wild type DNAs.

High efficiency of elaborated method was demonstrated in course of DRMTB cultures blind testing in 2005.

To investigate the spread of MTB drug resistance (rifampin and isoniazid) the screening of clinical culture samples of MTB from different Russian regions have been done. During monitoring we have studied MTB cultures strains from Russian regions including 1406 (70.2%) strains received from new-onset MTB patients and 596 strains from previously treated patients. Percentage of MDR-MTB strains received from new-onset patients was 21.9% and percentage of isoniazid-monoresistance strains from those patients was 13%. Percentage of MDR-MTB strains received from previously treated patients was 58.5% and percentage of isoniazid-monoresistance strains was 19.8%. The molecular genetic analysis of the scope and frequency of different codons and mutations in the genes of MTB resistant to rifampin and isoniazid was done.

TB-BIOCHIP® DIAGNOSTIC KITS FOR FAST IDENTIFICATION OF MTB COMPLEX AND DRUG SUSCEPTIBILITY TESTING

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REAGENTS AND TOOLS FOR RAPID TB SERODIAGNOSTICS IN RUSSIA

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Available diagnostic tools for diagnosis of TB are seriously limited by their duration, cost, technical complexity, difficulty in scale-up manufacturing, and unacceptable accuracy. The traditional laboratory test for diagnosis of TB infection includes sputum examination for the presence of *M. tuberculosis,* culture of sputum or other body fluid, the tuberculin skin test and radiology, which is either insensitive or time consuming. A few key tools or interventions, if developed, could therefore greatly assist existing control programs.

Among different diagnostic tools the immunochromatographic strip (ICS) in lateral flow format seems to be perspective. This test platform had attractive performance attributes for field and home application including use of relatively inexpensive off-the-shelf components and reagents, the ability to format the tests for detection of antigens or antibodies, and its usability with a wide range of specimens (e.g., exudates, swabs, urine, serum, plasma, or blood). In addition, once sealed in a pouch, the tests were stable for more than a year, could be shipped without refrigeration, and could be accurately performed and interpreted by minimally trained health care workers.

As consequence we are proposing to develop a one-step IC-immunoassay, which will be able specifically detect the antibodies to *M. tuberculosis* in human serum or plasma. At present time numbers of similar tests are being trials (for example Sequella) or already marketed. Developers of these tests usually take into account the fact, that in the several countries children are immunized by BCG. For prevent of false-positive reactivity serums, taken from BCG-vaccinated persons, it is used only such MTB antigens as absent in BCG. Because nucleotide sequences of BCG and MTB genomes are known, there were deciphered completely regions of distinction (RD). Based on these regions, ranges of tests on molecular genetics were proposed. A numbers of recombinant proteins with using ORF from RD were expressed in *E.coli* and other gene expression systems. Proteins received by this way were used as antigens for testing by patients serum, collected in different regions. Analysis of published data demonstrates that these antigens can interact with patients' serum in mosaic mode. The antigens possess different sensitivity ranging from 10 till 50%. At the same time, mixes or hybrids of several proteins' antigens are able to recognize more than 50% of positive serum. Ability of antigens to interact with negative serum (specificity) from healthy donors, including BCG vaccinated donors, varied from 5 till 30%. Our data, received during testing of the same 15 recombinant proteins, but with serums, collected from about 300 patients or 400 healthy donors in Central regions of Russia, revealed significant difference in antigens reactivity compared with published data.

One of mostly probable causes of observed differences is geographic specificity of antibody response at tuberculosis patients. The complicated phenomena of geographic specificity of antibodies response are commonly based on two key observations. At first, the MTB strains, circulating in distinct regions and countries, might have some antigens differentiation. At second, there exist national and ethnic peculiarities of immune response to infection, caused by the same strain of *M. tuberculosis.* It means that test has been constructed and validated on serum collected for example in South Africa will not demonstrate equal specificity and sensitivity in the case if serum samples were collected in Siberia or Uzbekistan. In other words, the received by this way test can be called "regional test". Taking into consideration such a situation, it is necessary to develop region-specific tests for achievement of maximum specificity and sensitivity.

It is important that during construction of this test we intend to consider geographic specificity (Central regions of Russia) of antibodies response to *M. tuberculosis*.

For that we will be use approach, based on preliminary testing of different kinds of TB antigens. The planned antigens are recombinant proteins, including glycosilated and chimerical, synthetic polysaccharides and their different combinations. Representative sampling of patients' and healthy donors' serum, collected in Central regions of Russia, will be done

EVALUATION OF THE TB TEST-KIT FOR RAPID DRUG SUSCEPTIBILITY TESTING OF *M. TUBERCULOSIS* IN MOSCOW SCIENCE AND PRACTICAL ANTI-TUBERCULOSIS CENTER

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Objective. To evaluate TB test-kit for susceptibility testing of *M.tuberculosis* to isoniazid, rifampicin, streptomycin and ethambutol in comparison with the absolute concentration method and the BACTEC 960 MGIT.

Methods. TB test-kit is a kit of ready-for-use nutrient media with drugs or without them and reagent Griess for reading results. The TB test-kit has been developed and introduced into manufacture in SRCAMB. Materials. 221 of *M.tuberculosis* cultures isolated on LJ medium from TB patients in Moscow Science and Practical Center of TB Control were used for trials. The drug susceptibility testing (DST) results obtained with TB test-kit were compared with those obtained by the absolute concentrations method and by the BACTEC 960 MGIT.

In the comparative trials the test-kit with the absolute concentration method TB test-kit had sensitivity for isoniazid of 98.0%, for rifampicin and streptomycin of 96.4%, and for ethambutol of 92.2%. TB test-kit specificity was 98,3% for isoniazid, rifampicin and streptomycin and 82.4% for ethambutol.

In the comparative trials the test-kit with the BACTEC 960 MGIT TB test-kit had sensitivity for isoniazid of 98,7%, for rifampicin 99,0 %, for streptomycin of 96.7%, and for ethambutol of 93.1%. TB test-kit specificity was 98,8% for isoniazid, rifampicin and streptomycin, and 84.5% for ethambutol.

The turnaround time for TB test-kit was 9,6 days (the range being 8-14 days), and the turnaround time for the absolute concentration method was 21,6 days (the range being 21-28 days), and the turnaround time for BACTEC 960 MGIT was 7,1 days (the range being 6-10 days).

The discrepant results analysis of discordant isolates was performed by the proportion method on Middlebrook 7H10 agar and by PCR-sequencing.

Conclusion. TB test-kit gives comparable results in the susceptibility testing to drugs of first line. The test-kit is not labor consuming, it does not require special equipment and skilled personnel, and, it is rapid and inexpensive.

DESIGN OF EXPERIMENTAL AEROSOL DNA VACCINE PREPARATION

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The lung represents a very attractive target for anti-hantaviral vaccination to achieve local (at the entry of the regular hantavirus infection) and systemic immune response. The objective of this study was to estimate the probability of construction an effective aerosol DNA vaccine (against hantaviral infection). The main problem in this approach is to protect the DNA when aerosolized in variety of generators and to provide effective penetration in deep respiratory segments. Our previous experiments with liposome DNA protection were not very useful, so we changed liposome for polyethylene imine, PEI.

Complexes of expression vector plasmid DNAs (cloned complete sequences of S, or M segments of hantavirus Seoul genome under CMV promoter) with PEI was prepared and the ultrasonic generator was used to transfer the complex into aerosol form to immunize animals (BALB/c mouse line) in special chambers with stable (for 0.5-1.0 hr) concentration and particle size distribution. These conditions provided more than 55% aerosol particles of less than 5μm and more than 90% particles of less than 10μm, and dosage per mice of 8-10μg / 0.5hr of intact DNA. Aerosol application was accompanied with intraperitoneal injection of adjuvant (complex proteoglycan of natural origin). The experiments were controlled adequately. Immunization scheme was the regular one: two priming applications and one boosting one with the same preparations in same doses. Samples of sera (and lung washes) were taken at 10-14 days after final application. Results were obtained in enzyme immune assays by appearance specific immune globulins of classes IgM, IgG and IgA. In 48-72 after each application lung tissue samples were analyzed in PCR for N gene sequence; it could indirectly prove the N gene expression. We succeeded to demonstrate good immune response when used aerosoled PEI:DNA+adjuvant. All three classes of immune globulins were presented in immunized mouse sera and lung washes.

These data suggest that aerosol DNA immunization may be a viable approach for designing of new antiviral vaccines.

RECOMBINANT PROTEIN BASED SKIN TEST FOR TB DIAGNOSTICS

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Intradermal Mantoux test (containing purified tuberculin) has been used for many years for TB infection (TBI) screening in population. Upon this testing due to the presence of general mycobacteria antigens, positive reaction does not distinguish between active TB form, sensibilization due to contact with *M. tuberculosis,* BCG vaccination or cross over sensibilization by other types of mycobacteria. Also testing often causes allergic reactions that complicate results interpretation. Demand for new TBI testing is obvious.
Reagents based on two secreted proteins *M. tuberculosis* ESAT-6 and CFP-10, that are not present in *M. Bovis* and in the majority of nonpathogenic mycobacteria are considered the most appropriate for this purpose.

Recombinant plasmid DNA (pTBD16) that codes the synthesis of hybrid protein CFP-10—ESAT- 6 *M. tuberculosis* was constructed for this product.

ZAO "Pharmaceutical firm "LEKKO"" produced this product – named "Diaskintest" that consists of recombinant protein CFP-10—ESAT-6, produced by E.coli BL21(DE3)/pCFP-ESAT.

Pre clinical trials have been completed according to Sanitary Rules and regulations by State Pharmaceutical Committee. The results showed that the product is non-toxic, does not cause sensibilization, save and specific – does not cause positive reaction in healthy animals and in animals vaccinated by BCG. Its specific activity can be compared to specific activity of purified tuberculin (PPD-L-2), which is a National standard (OSO). With the increase of TB caused damages in guinea pigs and delayed hypersensitivity, response reactions to the dilution of product are also increasing. The product detects the presence of TBI in guinea pigs a week earlier than traditional tuberculin.

Diaskintest has been tested on volunteers. Hyperergic, unusual, systemic or local side effects were not detected. Positive reaction to Diaskintest has been found in patients with active lung TBI, negative – in healthy individuals. Prognostic value of the products was higher than of the Mantoux test.

EFFICACY OF NANOPARTICLE-BOUND RIFAMPIN IN MICE INFECTED WITH *MYCOBACTERIUM TUBERCULOSIS*

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The objective of the present study was the development of poly (lactide-co-glycolide) polymer (PLGA) -based nanoparticulate formulation of rifampin and evaluation of its efficacy against acute tuberculosis infection in mice. The infected animals were treated with conventional (RIF) or nanoparticle bound (RIF-PLGA) rifampin using peroral and subcutaneous routs of administration using different weekly treatment schedules and dose 20 mg/kg of active compound. Untreated animals were used as control. To create a depot effect nanoparticles made from high molecular PLGA were used. The advantage of the nanoparticles could be seen for once-a-weekly treatment schedule. RIF is known as a highly effective antituberculosis drug with 95% bioavailability at peroral administration. Nevertheless, the application of the nanoparticles afforded the considerable increase of efficacy: CFU counts in lungs were decreased >5-fold in comparing with RIF. Again, the increased efficacy correlated with a higher RIF concentration and retention in lungs enabled by the nanoparticles. The results of the chemotherapy were supported by the pharmacokinetic data. We compared the drug biodistribution patterns after peroral and subcutaneous administration in healthy mice. In the case of subcutaneous administration, slightly higher concentrations were found in all organs for RIF, as compared to RIF-PLGA. In the case of peroral administration, nanoparticles afforded significant increase of lung and spleen concentrations. Interestingly, the plasma concentration profiles were similar for the RIF and RIF-PLGA. The mechanism by which perorally administered nanoparticles enable the enhanced RIF accumulation in lungs and spleen is not clear. However, these results demonstrate that PLGA nanoparticles are effective drug carriers for peroral administration of RIF. This approach may be especially useful for substances with low bioavailability. These results suggest that the nanoparticles bound with RIF have a certain potential for peroral application in the treatment of tuberculosis.

GENERATION AND MIGRATION OF $CD27^{lo}$ EFFECTOR CD4 T LYMPHOCYTES DURING MYCOBACTERIAL INFECTION IN MICE

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Tuberculosis remains one of the leading causes of morbidity and mortality by infection diseases worldwide. Protection against tuberculosis (TB) largely depends upon generation of CD4 effector T cells, which produce IFN-γ and activate infected macrophages. To develop improved strategies of TB control it is important to understand how IFN-γ-producing effector T lymphocytes are generated, distributed and regulated at the sites exposed to the pathogen. It was recently shown that $CD44^{hi}CD62L^{lo}$ effector CD4 T lymphocytes are heterogeneous and consist of $CD27^{hi}$ and $CD27^{lo}$ subsets. Only $CD27^{lo}$ subset exhibit a high level of IFN-γ secretion, indicating that the $CD27^{lo}$ phenotype is characteristic for fully differentiated effector CD4 T lymphocytes. In the present study we examined anatomical distribution and generation of $CD27^{lo}$ CD4 T lymphocytes during mycobacterial infection in mice.

Analysis of CD4 T lymphocytes recovered from lymph nodes (LN) and lungs of C57BL/6 mice infected with *M. tuberculosis* H37Rv demonstrated that $CD27^{lo}$ cells accumulated predominantly in the lungs but not in LN. Several lines of evidence support that preferential accumulation of $CD27^{lo}$ effector CD4 T cells in the lungs is due to their *in situ* differentiation from $CD27^{hi}CD62L^{lo}$ precursors. First, adoptive transfer of $CD27^{hi}CD62L^{lo}$ CD4 T lymphocytes resulted in the appearance of $CD27^{lo}$ cells of donor origin in the lungs of recipient mice. In contrast, donor cells recovered from lymphoid organs of recipients retain their $CD27^{hi}$ phenotype. Second, among lymphocytes newly migrated to the lungs the frequency of $CD27^{lo}$ cells was low, and the proportion of $CD27^{lo}$ cells steadily increased during their residence in the lungs. Experiments with LN cells labeled *in vivo* with CFSE and monitored regarding migration to mycobacteria-infected and uninfected lungs demonstrated that infection of lung tissue significantly promoted down-regulation of CD27. Finally, differentiation of $CD27^{lo}$ CD4 T lymphocytes from $CD62L^{lo}CD27^{lo}$ precursors was directly confirmed *in vitro* using purified $CD62L^{lo}CD27^{hi}$ and $CD62L^{lo}CD27^{lo}$ cells cultured *in vitro* in the presence of mycobacterial antigens. We observed gradual accumulation of $CD27^{lo}$ CD4 lymphocytes in cultures started with pure $CD27^{hi}$ cells. Altogether, our results suggest that lung $CD27^{lo}$ CD4 T lymphocytes differentiate *in situ* from $CD27^{hi}$ precursors.

To study a potential role of $CD27^{lo}$ effector CD4 lymphocytes in protection against TB, we analyzed accumulation of $CD27^{lo}$ lymphocytes in the lungs of mice that differ regarding susceptibility to TB infection. (A/Sn x I/St)F2 mice (N=106) originating from TB-susceptible I/St and TB-resistant A/Sn mice were infected with *M. tuberculosis* and monitored for the degree of body weight loss. The degree of wasting oppositely correlated with the frequency of $CD27^{lo}$ CD4 T cells, suggesting that efficient transition of $CD27^{hi}$ into $CD27^{lo}$ effector lymphocytes contributes to protection.

SYSTEM FOR SUPEREXPRESSION OF TUBERCULOSIS VACCINE IN PLANT

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Recent development of genetic engineering allowed employing the plants as factories for the foreign proteins production. Thus, tuberculosis (TB) antigens were expressed in different plant systems, but the level of vaccine protein accumulation was extremely low. Here, we describe the system for superexpression of TB vaccine proteins (Ag85B, ESAT6, and ESAT6:Ag85B fuse) in *Nicotiana benthamiana* leaves which involves: (i) Construction of crucifer infecting tobacco mosaic virus- and potato virus X-based vectors with the coat protein genes substituted by those of TB antigens; (ii) *Agrobacterium*-mediated delivery to plant leaf tissues of binary vectors containing the cDNA copy of the vector virus genome; (iii) developed novel methods of silencing suppression; (iv) addressing TB antigens in different cell compartments and cell surface (apoplast); (v) TB antigen coding sequence modifications allowing a dramatic stimulation of TB vaccine protein intracellular stability. This technology enables efficient production of the TB vaccine proteins in plant; in particular, the level of Ag85B antigen accumulation was not less than 1 g/kg of fresh leaves. Expression of TB antigens in plant cells as His_6-tagged proteins promoted their isolation from cell fractions and purification by Ni-NTA affinity chromatography. Another way of purification is using apoplast liquor of leaves agroinjected with cDNA expressing TB antigen fused with tobacco cell wall pectin methylesterase signal sequence. We propose that the strategy of TB antigens superproduction in a plant might be used as a basis for creation of prophylaxis and therapeutic vaccine against TB.

ACCELERATED DETECTION OF MTB CELLS FOR DIAGNOSTICS OF TUBERCULOSIS BASED ON RESUSCITATION OF DORMANT CELLS AND THEIR IMMUNE DETECTION

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The work is directed on the elaboration of the analytical system for accelerated (7-10 days) detection of active and dormant *Mycobacterium tuberculosis* (MTB) cells in biological samples. All existing bacteriological methods do not allow revealing sub-populations of dormant) or injured cells which can form considerable part of mycobacteria in clinical samples. The proposed approach includes reactivation of dormant/injured cells, their subsequent rapid growth and specific immunological detection. By this way, advantages of immunological and bacteriological analyses are combined. The analytical system includes test tube with a special media containing growth factors and immunochromatographic test-strip contacting with the media on the final stage of the analysis. Application of colloid gold as immunochromatographic marker excludes necessity in additional devices and reagents to carry out immunoassay and provides possibility of visual registration of the assay results.

To realize microbiological part f the test-system, family of Rpf proteins was characterized as inductors of mycobacteria transition from dormant to active form. The improved protocol of recombinant Rpf protein obtaining from *M.luteus* (RPF-L) was elaborated; it is characterized by high yield of the product (0.5-0.8 mg from 1 L of media). Biological activity of the RPF-L was tested in terms of stimulation of the *M.luteus* growth in stationary stage. In all of the experiments RPF-L reduced lag-phase of the test-culture growth in several times, that allows to use this protein for accelerated determination of mycobacteria. Experiments with MTB cells having deletions of three Rpf genes demonstrated that the addition of recombinant Rpf proteins into growth media causes partial resuscitation of the cells. Maximal reviving activity was shown by MTB cultural liquid in logarithmic phase of growth. Thus, proper supernatants can be used as an additive to the cultural media for maximal revealing of dormant MTB cells.

To reach maximal sensitivity and selectivity of immunochromatographic analysis in sandwich format, we have offered to use a combination of antibodies against individual *M.tuberculosis* proteins and polyclonal antibodies against total cellular antigen. At optimized conditions of competitive microplate immunoenzyme assay the chosen antisera provide possibility to detect 10^4 cells/ml. To realize immunochromatographic assay, gold colloidal carriers of different size (from 5-6 to 30 nm) were obtained. Protocols of their conjugation with specific anti-MTB antibodies were optimized to reach monolayer formation, thus providing stability of the formed conjugates. Immunochromatographic assay of MTB cells was realized in competitive and sandwich formats with detection limits 10^4 and $2*10^5$ cells/ml, correspondingly.

The obtained results create necessary basis for the creation of the new system for accelerated diagnostics of tuberculosis.

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NEW APPROACHES TO THE TUBERCULOSIS THERAPY USING RECOMBINANT CYTOKINES AND SYNTHETIC PEPTIDES

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Tuberculosis (TB) is a chronic infectious disease which worldwide afflicts approximately 30% of the world's 5.7 billion people. More than 10% of the infected persons will progress to fulminant TB by early adulthood. More than 3 million people die of TB each year; TB kills more people today than does any other infectious agent. That is why development of new therapeutic approaches for treatment of TB is of great importance. One of these methods include immunotherapy with recombinant cytokines and immunostimulatory synthetic peptides. Among them human recombinant interleukin-1 beta has been shown to be highly effective additive to standard tuberculosis chemotherapy in clinical practice.

The response of the human immune system to antigens, including infectious agents, is directed by two different subsets of T-helper cells (referred to as type 1 and type 2, or Th1 and Th2). Evidence has accumulated that in mycobacterial infection, it is the CD4+ Th1 cells and their associated cytokines (IL-2 and IFNγ) which play a key role both in protective immunity and immunopathology. is considered to be the major macrophage activating factor, which also plays a major role in suppressing a potentially disease-promoting Th2 immune response.

SCV-07 (gamma-glutamyl-tryptophan) is one of a group of new immunodulatory compounds that possess gamma-glutamyl or beta-aspartyl moieties, which were developed and patented both for composition and immunomodulatory use. The most potent analog, SCV-07, has been shown to have a broad spectrum of immunostimulatory activities both *in vitro* and *in vivo*. As SCV-07 injections in mice immunized by protein antigen cause increased production of Th1 cytokines such as IL-2 and IFNγ, but not Th2 cytokines, it could be proposed that SCV-07 stimulates the immune response through preferential activation of the Th1 lymphocyte subset. SCV-07 is thus well suited for therapy of infectious diseases connected with an inadequacy of Th1-mediated immunity, such as TB. The biological activity of SCV-07 was investigated in a model of induced experimental tuberculosis. SCV-07 had evident antituberculosis activity that was evaluated using the experimental tuberculosis severity criteria such as macroscopic examination of lungs and spleen, and quantitation of lung damage, spleen weight and lung weight. In addition, the animal's weight and mycobacterial culture from spleen were analyzed. SCV-07 clinical trials in patients with lung tuberculosis showed its clinical efficacy and immunomodulatory properties.

APPLICATION OF MOLECULAR DIAGNOSTIC TOOLS FOR MONITORING OF TREATMENT AND TESTING OF THE NEW PHARMACEUTICAL COMPOSITION FOR ADDITIONAL CHEMOTHERAPY OF TUBERCULOSIS

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Tuberculosis is a worldwide health problem and may be the most common cause of deaths from a single infectious agent. The conventional method for detection of *Mycobacteria is* in clinical samples is based on the demonstration of the acid-fast organisms following cultivation. This method is reliable but time-consuming (6-8 weeks) and needs more sensitivity.

Polymerase chain reaction (PCR) and Real time PCR are a simple, sensitive (several copy of the bacteria) and rapid tests, useful in detecting of *M. tuberculosis* complex. We have used PCR and Real time PCR for qualitative and quantitative detection and differentiation of *M. tuberculosis* and *M. bovis* in cultures, blood, animal tissues and human clinical samples using more then 3000 samples. There are more then 2000 kits for detection and differentiation of *M. tuberculosis* and *M. bovis* based on PCR and Real rime PCR have been producing for veterinarian clinical laboratory.

We have been demonstrated gene expression of *Mycobacteria* using RT-PCR on the RNA samples for detect of viability of the bacteria. More then 200 clinical samples from 80 patients with pulmonary tuberculosis and 146 samples of spinal liquid and blood from patients with tubercular meningitis during 10 months on different stages of therapy have been tested.

Efficiency of the therapy has been tested using molecular methods and classical methods (bacteriology and microscopy). Detection DNA and mRNA was important for analysis of viability of bacteria before and after treatment. The schema for interpretation of molecular analysis has been proposed.

Expressions of the Multidrug resistance MDR-1 gene in treated tuberculosis patients have been studied. More the 150 from Moscow City Research Center for Tuberculosis Control, Moscow Health department have been examine for MDR-1 gene expression. Expression of MDR-1 gene has been detected in the samples from patients having problems with treatment has been shown.

The new pharmaceutical composition ("Mycopharma") for additional chemotherapy of tuberculosis has been developed. The pharmaceutical composition prevents to propagation of *M. tuberculosis* in human organism and at the same time raises immunity of during different stages immune-response.

The object of this proposal is to application of the complex of molecular diagnostics for fundamental and practical research in field of tuberculosis.

The goal of this project is to develop and design of the new schemes and recommendation for differential diagnostics, monitoring of treatment and testing of the new pharmaceutical preparations for tuberculosis based on molecular methods.

In order to achieve the main objective, the following specific aims are proposed

Fundamental research regarding kinetic of propagation of the tuberculosis in the human organism (latent, steady-state, and post-stationary phases).

Monitoring of efficiency of anti-tuberculosis treatment for each phases.

Development of the new schemes for clinical molecular diagnostics of tuberculosis for patients from different hospitals.

Development of the new schemes for molecular diagnostics of tuberculosis for animal in different farms.

Testing of the efficiency of new pharmaceutical composition for additional chemotherapy of tuberculosis using of molecular methods.

Research of the multidrug resistance (MDR-1) gene expressions in treated tuberculosis patients for developed additional methods for assessment of the efficiency of anti-tuberculosis therapy.

It is expected that the implementation of the research will provide new data about the interaction between mycobacterial and human's or animal's organisms. We expect also that the project will lead to the development of new schemes of molecular diagnostics of *Mycobacteria* for qualitative and quantitative detection, differentiation and monitoring of the treatment. The development of diagnostic reagents may be of commercial significance, but their direct expected effect will be associated with the improvement of the program for control for tuberculosis in hospitals and farms in Russia, implemented by the Russian Ministry of Health and the Russian Ministry of Agricultures.

IMMUNOPATHOLOGY OF A PULMONARY TUBERCULOSIS: FUNDAMENTAL AND APPLIED ASPECTS

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Last years signs negative clinical pathomorphosis of tuberculosis are found out. High frequency of registration of widespread destructive forms of an infection and decrease in efficiency of specific therapy testifies to it. One of the most probable reasons of such dynamics evolution of pathological properties Mycobacterium tuberculosis (MBT) is considered. Along with it, biological properties of the MBT are important, but not unique factor defining development of disease. The condition of immune system renders not less (and, probably, and more) essential influence on current character and outcomes of tuberculosis.

The estimation of a territorial origin and degree of genetic relationship of MBT is made. It is shown, that in territory of Tomsk and Tomsk oblast circulate genetically closely related MBT strains of 47 VNTR-types, the high clasterization indicator which (65.66%) testifies to active transmission of the MBT. It is established, that the genotype infecting strain of MBT defines not only features of a clinical current of a pulmonary tuberculosis, but also substantially affects features of immune cells reaction in the course of a tubercular inflammation. Prevalence allelic variants of genes cytokines (*NRAMP1, IL1B, IL1RN, IL12B)* at Russian inhabitants of Tomsk is investigated. The complex estimation of molecular mechanisms immune disbalance is lead at infiltrative, disseminative, fibrous-cavernous pulmonary tuberculosis with sensitivity and multiple drug-resistance of MBT to antitubercular chemotherapy, including research of immunophenotype, cytogenetic, morphological, metabolism statuses, stimulated proliferative, secretive activity and apoptosis of blood immunocytes prior to the beginning and in dynamics of antitubercular therapy. Key pathogenetic factors of dysregulation of B- and the T-link of immunity, accompanying a current of a pulmonary tuberculosis with sensitivity and multiple drug-resistance of MBT are allocated. It is shown, that at the base of defect of the T-cellular link of the owner at drug-sensitive and drug-resistance variants of an infection lay as similar (oppression of proliferation, activation of apoptosis), and differing mechanisms: in the first case - is free-radical damage of lymphocytes, in the second - mediated by cytokines and apoptosis removal reactive T-cells. It is revealed, that the pathology of the T-link of immunity at drug-sensitive pulmonary tuberculosis is not absolutely decompensated, is developed during infections and characterized as «immune damage against immune irritation». The base of T-deficiency at drug-resistant pulmonary tuberculosis is immune tolerance mediated by the MBT.

Search of factors of the activator and the owner predetermining occurrence, current and the adverse forecast of a tubercular infection is conducted. The polymorphism contribution genotypic properties of MBT and the genes causing activation and suppression of adaptive immunity, in formation of various clinic and pathogenetic variants of a widespread destructive pulmonary tuberculosis is found out. Associations of +3953A1/A2 polymorphism of *IL1B* gene with the limited destructive pulmonary tuberculosis, VNTR polymorphism of *IL1RN* gene and 274S/T polymorphism of *NRAMP1* gene with the widespread destructive form of disease, 1188A/C polymorphism from *IL12B* gene both with a widespread destructive pulmonary tuberculosis, and with primary on genesis the pathology form are revealed.

The obtained data are represented important for formation of representations about interrelation of features genetic parameters of population MBT and the person with pulmonary tuberculosis pathomorphism, allow to get more deeply into fundamental mechanisms of a variation of clinical displays and immunopathogenesis of a tubercular inflammation and can be used for organization of preventive and medical actions, including directed personalized immunocorrective therapies.

INFORMATIVITY OF GENETIC AND PHARMACOKINETIC TESTING IN PREDICTION OF ANTITUBERCULOSIS DRUGS INDUCED HEPATOTOXICITY

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The high frequency of adverse reactions including hepatotoxicity are a concomitant result of the intensive antituberculous treatment. Estimation of patient individual genetic features in antituberculous drugs metabolism is assumed as a perspective approach to decrease a risk of these reactions. However discordance of genotype and phenotype in drug metabolism is possible. We investigated the possibility of the hepatotoxicity prediction in patients receiving isoniazid, rifampicin, pyrazinamide and ethambutol on the basis of isoniazid pharmacokinetics and genetic polymorphism of arylamine N-acetyltransferase 2. There were detected 481C>T, 590 G>A and 857G>A SNP's in 75 patients with lung tuberculosis and in 52 patients pharmacokinetics of isoniazid in first days of therapy was estimated. Distribution of pharmacokinetic parameters of INH elimination (constant of elimination, clearance total and time of half-elimination (t1/2) was bimodal with antimodes 0.2 h^{-1}; 3.5 ml/min/kg and 3.2 h, respectively. Median values of t1/2 were equal 6,44 h for slow and 1,8 h for rapid acetylators. Frequencies of 481T, 590A and 857A alleles were 0.36, 0.29 and 0.06, respectively. In the group of examined patients there were 13 cases (26%) of inconsistency between genetically predicted and pharmacokinetically determined acetylation phenotype, 11 of them were dislocations of genetically fast acetylators into phenotypically slow acetylators. Levels of alanine aminotransferase (ALT) were elewated during first month of therapy in genetically as well as pharmacokinetically determined slow acetylators. However level of statistical significance of differences between initial values of ALT with those after 1, 2 and 3 month of therapy was higher when pharmacokinetic data were analyzed. Thus results show preferability of pharmacokinetic estimation for individual prognosis of drug-induced hepatotoxicity.

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