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IMMUNOLOGIC MEMORY REVIEW

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The first results of the clinical trial of GamTBvac recombinant vaccine in healthy volunteers

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Effect of high cyclophosphamide doses on the repertoires of peripheral blood T-cell receptors

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НАУЧНЫЙ МЕДИЦИНСКИЙ ЖУРНАЛ РНИМУ ИМ. Н. И. ПИРОГОВА

ДЛЯ ПОДАЧИ РУКОПИСЕЙ editor@vestnikrgmu.ru
ДЛЯ СОТРУДНИЧЕСТВА manager@vestnikrgmu.ru
АДРЕС РЕДАКЦИИ ул. Островитянова, д. 1, г. Москва, 117997

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IMMUNOLOGICAL MEMORY AS A BASIS FOR A WISE VACCINATION STRATEGY. A RATIONALE FOR INTRODUCING A COMPREHENSIVE SEROEPIDEMIOLOGICAL SURVEILLANCE SYSTEM IN RUSSIA

Gushchin VA[1,2] ✉, Manuilov VA[1,3], Mazunina EP[1], Kleymenov DA[1], Semenenko TA[4], Gintsburg AL[5], Tkachuk AP[1]

[1] Laboratory of Translational Biomedicine,
N. F. Gamaleya Federal Research Center for Epidemiology and Microbiology, Moscow, Russia

[2] Department of Virology, Faculty of Biology,
Lomonosov Moscow State University, Moscow, Russia

[3] Institute of Molecular Medicine,
I. M. Sechenov First Moscow State Medical University, Moscow, Russia

[4] Department of Epidemiology,
N. F. Gamaleya Federal Research Center for Epidemiology and Microbiology, Moscow, Russia

[5] N. F. Gamaleya Federal Research Center for Epidemiology and Microbiology, Moscow, Russia

Immunological memory is one of the key features of the adaptive immunity. It confers the ability to resist infection and prevents development of cancer or autoimmune diseases. Most importantly, immunological memory mediated by preexisting antigen-specific clones of T- and B-cells ensures a rapid and effective response to an invasion by a previously encountered pathogen. Since vaccination induces a specific long-lived response to infectious agents, it becomes a basis for preventive medicine. In a human population, immunological memory of individuals shapes the so-called herd or community immunity crucial for national health. The present review touches upon significant population-wide research studies of immunological memory with regard to immunization. We discuss the principles of serological testing and the outcomes of serological monitoring conducted in different countries, and talk about standard and innovative analytical approaches to studying immunological memory. We also pinpoint the drawbacks of methods used for herd immunity assessment in Russia and propose a comprehensive system for seroepidemiological surveillance.

Keywords: immunological memory, herd immunity, humoral immunity, infectious agent, pathogen, vaccine, immunization, immunization schedule, vaccine hesistancy, surveillance

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✉ **Correspondence should be addressed:** Vladimir Gushchin
ul. Gamalei, d. 18, Moscow, Russia, 123098; wowaniada@gmail.com

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ИММУНОЛОГИЧЕСКАЯ ПАМЯТЬ КАК ОСНОВА РАЦИОНАЛЬНОЙ ВАКЦИНОПРОФИЛАКТИКИ НАСЕЛЕНИЯ. ОБОСНОВАНИЕ СОЗДАНИЯ СИСТЕМЫ СЕРОЭПИДЕМИОЛОГИЧЕСКОГО МОНИТОРИНГА В РОССИИ

В. А. Гущин[1,2] ✉, В. А. Мануйлов[1,3], Е. П. Мазунина[1], Д. А. Клейменов[1], Т. А. Семененко[4], А. Л. Гинцбург[5], А. П. Ткачук[1]

[1] Лаборатория трансляционной биомедицины,
Национальный исследовательский центр эпидемиологии и микробиологии имени Н. Ф. Гамалеи, Москва

[2] Кафедра вирусологии, биологический факультет,
Московский государственный университет имени М. В. Ломоносова, Москва

[3] Институт молекулярной медицины,
Первый Московский государственный медицинский университет имени И. М. Сеченова (Сеченовский университет), Москва

[4] Отдел эпидемиологии,
Национальный исследовательский центр эпидемиологии и микробиологии имени Н. Ф. Гамалеи, Москва

[5] Национальный исследовательский центр эпидемиологии и микробиологии имени Н. Ф. Гамалеи, Москва

Иммунологическая память — одна из ключевых функций адаптивного иммунитета, позволяющая организму противостоять инфекциям, предотвращать развитие онкологических и аутоиммунных заболеваний. Важнейшее свойство иммунологической памяти — это способность с помощью предсуществующих антиген-специфичных клонов B- и T-клеток быстро и эффективно реагировать на патоген, с которым у организма был контакт в прошлом. Наличие долгоживущих популяций клеток, надежно распознающих инфекционные агенты, позволяет использовать вакцинацию в качестве главного элемента превентивной медицины. На уровне сообщества людей иммунологическая память отдельных индивидуумов определяет феномен популяционного (коллективного) иммунитета, который чрезвычайно важен для обеспечения биобезопасности в масштабе государства. В обзоре описаны актуальные исследования популяционного иммунитета в контексте вакцинопрофилактики. Рассматриваются принципы организации серологических исследований, опыт серологического мониторинга в различных странах, существующие и перспективные аналитические подходы к изучению иммунологической памяти. Обсуждаются недостатки принятых в России способов оценки популяционного иммунитета и предлагается к реализации система сероэпидемиологического мониторинга.

Ключевые слова: иммунологическая память, популяционный иммунитет, гуморальный иммунитет, инфекционный агент, патоген, вакцина, вакцинопрофилактика, календарь прививок, отказ от вакцинации, мониторинг

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✉ **Для корреспонденции:** Гущин Владимир Алексеевич
ул. Гамалеи, д. 18, г. Москва, 123098; wowaniada@gmail.com

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Vaccination is a powerful and cost-effective tool for fighting infection. It confers direct protection against pathogens and establishes a long-lasting immunological memory [1]. In spite of significant achievements in preventive vaccination, development of novel or improved vaccines is hampered by the lack of fundamental immunological knowledge, excessive bureaucratic regulations, financial and sociopolitical barriers, which also contribute to the irrational use of older preparations. For example, about 20 million children currently do not have access to routine vaccination (see the Figure), which entails deaths of several million people each year [1]. Even more infected people die because there are no vaccines against the pathogen (HIV, malaria) [2] or because the vaccine is only partially protective (tuberculosis) [3].

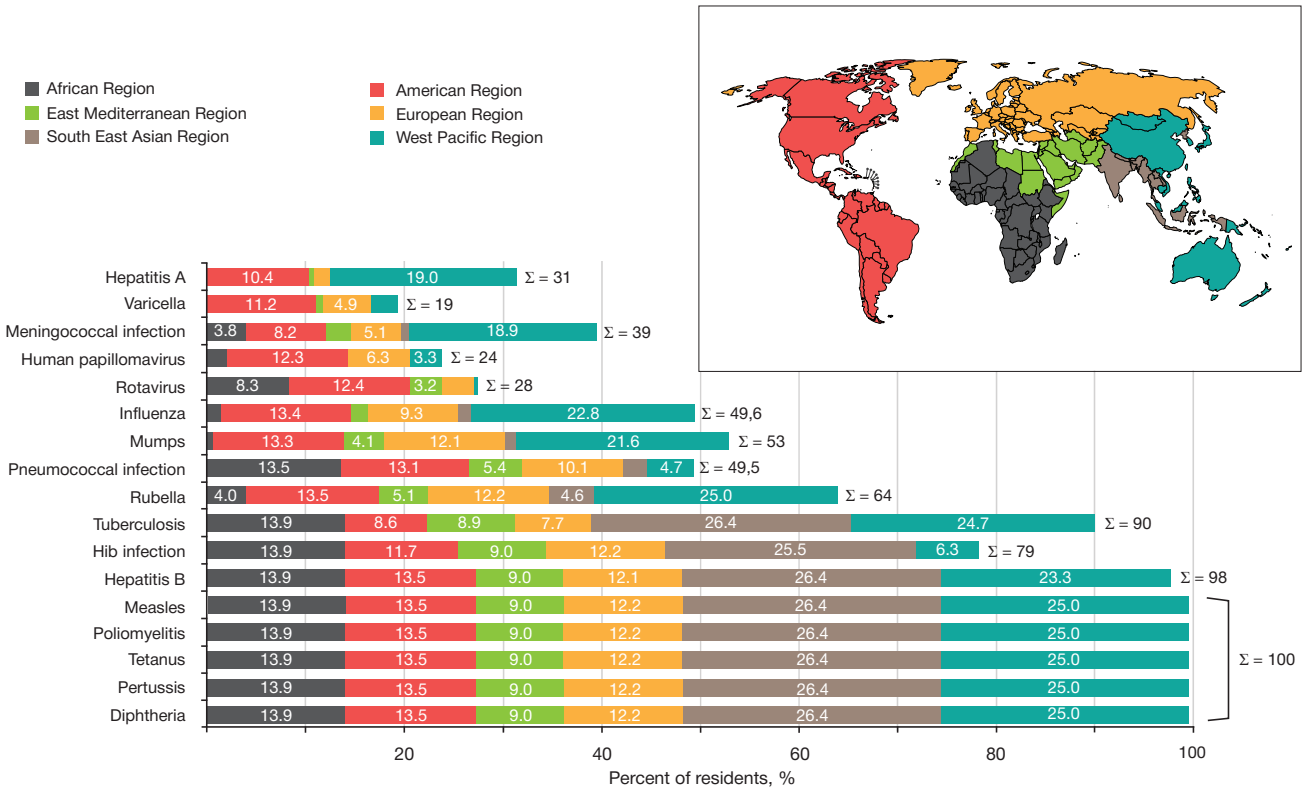
Until the 1970s, the list of recommended vaccines against life-threatening infections was quite short, and preventive vaccination basically aimed to cover the entire population regardless of age. Among the greatest achievements of that time was complete eradication of smallpox and partial elimination of measles, polio, diphtheria, tetanus and tuberculosis (reported in a few developed regions in Europe and North America). Current immunization schedules for children, teenagers and adults are increasingly intricate and offer vaccinations against multiple pathogens with dozens of serotypes.

Immunization schedules ensure the best use of available vaccines and also serve to accurately estimate the need for vaccination among different population groups. The list of recommended vaccines is constantly expanding, further complicating the immunization schedule. Still, the latter cannot be revised unless we start to monitor the efficiency of current vaccination programs and obtain a deeper understanding of herd immunity structure [5].

It is known that immunity of those who have developed immunological memory against a particular pathogen (through vaccination or natural infection) protects susceptible members of the population from this infection. This phenomenon is commonly referred to as herd immunity. Threshold proportions of immunes necessary for establishing herd immunity against a particular infection vary depending on the number of individual contacts in the population and the probability of infection transmission which, in turn, depends on the pathogen. For example, the spread of diphtheria, polio and rubella can be controlled if the proportion of immune individuals evenly distributed in the population is 82 % to 87 %; with mumps, the threshold value is 85 % to 90 %, and with measles and pertussis, this proportion is 92 % to 95 % [6].

It should be noted that a hundred percent immunity against infection is nonexistent: not every healthy individual develops a long-lasting post-vaccination immunological memory [7]. So far, factors determining this selectivity, including patient's genotype, remain understudied [8]. Also, particularly vulnerable to infection are patients with compromised immunity, HIV, or cancer [9]; infants in whom vaccination can be ineffective or unsafe [10], and elderly people with waning immunity [11]. Besides, even trustworthy vaccines sometimes differ in their effectiveness depending on the lot or season [10, 12]. Therefore, maintaining a high proportion of truly immunized individuals is crucial for the population in general and its vulnerable members in particular.

Importantly, herd immunity can be established only if immunized individuals are distributed evenly in the population [5]. It is known that geographical and social clustering of vaccine-hesitant groups, e.g. religious communities, seriously reduces herd immunity in the regions of their residence. This phenomenon has been demonstrated in the Mennonites (USA), indigenous peoples of the US and Canada, and religious communities in the Netherlands [6, 13]. Increasing vaccine hesitancy both in Russia and worldwide has become a high-profile issue [14, 15] and sparked serious discussions among the scientists [16–20]. Understanding the landscape



Percent of World Health Organization (WHO) member states' residents relative to the total population of WHO member states which include immunization against major vaccine-preventable infections in their immunization schedules (according to WHO [4])

of population immunity is critical for better identification of susceptible individuals and requires targeted serological surveillance.

Serological surveillance is an important tool used to monitor infection, identify at-risk populations and predict new threats. It provides information that is often missing from epidemiological and vaccination reports [20]. Modern serological surveillance relies on quantitative and qualitative measurements of specific antibodies in the blood serum that indicate post-vaccination or naturally acquired immunity and protect an individual from infection [21]. Antibodies generated in response to disease point to clinical or even subclinical infections that otherwise could be overlooked. For example, 20 % of infants with no medical history of pertussis and no previous vaccinations against this infection were found to have antibodies to the pertussis toxin [22]. Centralized serological surveillance of population immunity should be a part of any large-scale vaccination campaign.

Basic principles of serological surveillance

Any state-supported system for serological surveillance [23, 24] aims to obtain reliable data to estimate herd immunity, assess efficiency of current immunization programs and the need to revise immunization schedules and vaccine procurement volumes. Therefore, such a system should:

1. determine the actual proportion of individuals with protective titers against pathogens circulating in endemic areas, including infections that can be controlled by specific prevention measures, using appropriate stratification if required;
2. assess efficiency of current immunization programs from regional, socio-economic and age perspectives by comparing vaccination coverage statistics with the actual figures on the truly immune population;
3. update the statistics on herd immunity in population groups or areas (herd immunity refers to the percent of immune individuals in a studied population, sufficient to prevent an epidemic).

The main criteria a comprehensive serosurveillance system should meet are quality and quantity of the obtained data ensured by adherence to the following principles [25, 26]:

– *adequate coverage*: surveillance should be performed over the targeted population or environmental objects critical for accurate estimation of prevalence of infection markers (depending on the pathogen type, its endemicity, modes of transmission and risk factors); methods of statistical analysis should be applied;

– *relevance*: samples should be representative of the studied group or object; studies should be conducted in accordance with the principles of modern epidemiology;

– *continuity*: continuity of surveillance, i.e. a possibility to reproduce a study over the course of a few years;

– *data homogeneity* implies that data are homogenous enough to be pooled for further analysis within a given epidemiological and time frames.

A serosurveillance system would be impossible without authorized experts whose primary task is to collect, process and archive the samples using quality lab equipment and their own data processing centers. The need for a serosurveillance system was plainly indicated by the Russian Federation Government [23] and stems from the current state of the field.

Current methods for estimating herd immunity in Russia

At present, no large-scale studies are being conducted in Russia to estimate the prevalence of serological markers of vaccine-preventable infections whose results could be reliably extrapolated onto the entire population. Effectiveness of immunization programs is normally indirectly assessed by

– analyzing documented vaccination coverage rates;
– analyzing infection incidence rates,
– local seroepidemiological studies.

We will enlarge on these methods below.

Vaccination coverage

Every authorized vaccination provider is expected to fill out two statistical report forms [27, 28]: Form 5 that reports the absolute number of administered vaccine doses, and Form 6 that lists population groups eligible for vaccination before reaching the certain (decreed) age stated in the National immunization schedule [29, 30]. Based on these forms, the following parameters are calculated: *vaccination coverage among children*, i. e. the proportion of children who have reached a recommended age for vaccination and thus received at least one vaccine from a full vaccination course; *total vaccination coverage*, i. e the proportion of individuals of all ages who have received a full vaccination course; *timeliness*, i.e. the proportion of children who received their vaccines on time, i. e. before turning a certain age (see [31] for details). These reports are then collected by Rospotrebnadzor (the Russian Federal Service for Surveillance on Consumer Rights Protection and Human Wellbeing) [32, 33], and immunization coverage data get included into the Annual State Report [34].

This approach accounts for every country's resident who has received a vaccine, but does not account for those who have not received it. First, to calculate the proportion of vaccinated individuals, one should know the exact size of a population subgroup, but healthcare facilities (HCFs) that administer vaccines to their patients only have information about those who are registered with the facility (the so-called census population) [33]. Migration and failure to register with a local practice render these data unrepresentative of the actual size of different subpopulations in the society. Besides, in the absence of a single unified database of electronic medical records, information about vaccines administered to an individual is lost once they move to a different area. Second, even the most accurate estimates of patients vaccinated during the year are not representative of the size of the population vaccinated at any time point in their life. For example, the work by Tsvirkun [35] reports the rates of measles vaccination coverage in children from 1968 to 2013. In the late 1960s immunization coverage was 30–50 %, by the end of the 1980s it had reached 75 %, and by 2013 — 97 % to 98 %. In spite of seemingly inspiring immunization coverage, mathematical modeling predicted [35] that by the year 2014 the proportion of susceptible individuals would be as high as 25.6 %. Of 74.4 % of immune individuals, 50 % were previously vaccinated, while other 24.4 % acquired immunity after the disease. These figures differ drastically from thresholds necessary for measles eradication [36–39].

Another example of vaccine preventable infection is hepatitis B. The federal hepatitis B immunization program for newborns was launched in Russia in 2001 [40, 41]. Unfortunately, details on the immunization coverage in infants before the year 2010 have never been published, but between years 2010 and 2016 the rates were about 97 % [42]. By comparing these figures to birth rates in that period (about 2 million births a year [43]), one can assume that at least 14 million children have been vaccinated against hepatitis B so far, accounting for 10 % of the country's population. But such high immunization coverage does not provide any information about the total number of

Russians who have received the vaccine by now. First, we do not know how many people had become immune by 2001 (either through vaccination or after the disease) and how many newborns were vaccinated between 2001 and 2009. Second, there is only scarce data available on vaccinations between 2006 and 2008 [44, 45], arguing that 18.5 to 25 million adults, teenagers and infants were vaccinated outside the immunization schedule. These data are not sufficient to infer the number of Russian residents who have already received a vaccine against hepatitis B and/or have immunity to this disease.

Finally, vaccination does not always mean immunization [7, 10, 12]. Therefore, documented immunization coverage cannot be regarded as a criterion to estimate herd immunity.

Analysis of infection incidence rates

Incidence is defined as a proportion of new cases of infection within a period of time with respect to the total population [46]. Every case gets listed in the expedited report No. 058/U [47, 48] and in the statistical report Form 2 [27]. These forms are sent over to Rospotrebnadzor [32, 33] for summarizing and publishing [34]. Incidence rates are an objective criterion for public health, which is determined, among other things, by immunization coverage and effectiveness. However, incidence rates cannot be used to measure herd immunity for a number of reasons.

First, only new cases of infection are registered, specifically in patients who decided to seek advice of a doctor. Therefore, the actual incidence remains unknown. Moreover, a cohort of patients who regularly visit a doctor may be absolutely unrepresentative of the entire population [25].

Second, a number of infections require laboratory confirmation for diagnosis. This diagnostic step is very likely to be skipped if a local primary care facility is poorly equipped [49]. Good examples here are flu and acute viral respiratory infection. According to the official statistics, in 2016 flu afflicted only 88.5 thousand Russians (0.06 % of the population), while AVRI of unknown etiology — 31.7 million people (about 20 % of the population) [34]. Similar incidence rates were reported earlier [42, 50]. In contrast, as many as 22–29 million US residents (7 % to 9 % of the population) were diagnosed with flu during the non-epidemic season of 2015-2016 [51], while in Japan the annual incidence of flu was 10–16 % in 2010–2014 [52–54]. A clear disparity between flu and AVRI incidences typical for Russia is explained by the fact that AVRI can be diagnosed based on the symptoms, while flu diagnosis requires laboratory confirmation [55]. Flu is a vaccine-preventable infection, while AVRIs are not; therefore, underreported flu incidence prevents us from assessing the success of immunization programs.

Third, for some infections the number of new cases only loosely correlates with their prevalence. For example, hepatitis B is manifested either as a chronic or acute condition [56]. According to the official statistics, the incidence of acute hepatitis B decreased from 141 cases per 100,000 people in 1999 to 22.7 cases in 2016, which was largely due to the immunization program for newborns and the successful war on drugs [57, 58]. The total number of Russians infected with hepatitis B was determined by screening various population groups for the presence of HBsAg in the blood serum and accounted for 2 % to 4 % of the population (3-6 million virus carriers). This percentage seems to be stable over the years [59–62] due to the epidemiological specifics of virus dissemination [63]. The changing number of new registered hepatitis B cases does not correlate with the actual incidence of the infection in the population and is a poor marker of herd immunity against this disease.

An important metric related to incidence is mortality; however, in Russia the method used for calculating mortality may distort the resulting data. WHO estimates that infections account for 25 % of total deaths worldwide and for 10 % of deaths in the developed countries [2]. But according to the official statistics [43], between 2010 and 2014 only 1.7 % to 3.1 % of deaths were caused by infections in Russia [64]. The reason is simple: many fatal tumors (especially gynecologic cancers), gastrointestinal and cardio-vascular diseases are caused by infections, but it is impossible to reliably identify the cause of the disease in a person who dies of its complications or co-morbidities [65]. This means that data on infection-related deaths are inaccurate and cannot be used to estimate herd immunity.

Seroepidemiological studies

In Russia, there is a state-supported system for serological surveillance of vaccine-preventable infections in the target populations [31, 33, 36], operating via regional centers for hygiene and epidemiology of Rospotrebnadzor and core research facilities [35, 55]. This system, however, is not intended for studying herd immunity, but rather for investigating resistance to a particular infection [67] in vaccinated individuals representing differently aged sentinel populations [68]. This information is essential when assessing vaccination effectiveness, but such type of surveillance does not allow studying herd immunity of the entire country's population.

A wealth of information could be provided by population studies of prevalence of serological markers (including antibodies to pathogens) conducted by research institutions on their own initiative. Unfortunately, such studies are rare, carried out in small populations, published in different journals, and do not build a holistic picture of herd immunity in Russia.

Below are a few examples of such research works. The study [69] demonstrated that only 60 % to 78 % of 300 Leningrad oblast residents (the proportion varied depending on the age group) had antibodies to rubella. The samples used in that work were collected back in 1995, before the state-funded rubella vaccination program was launched in 2000 and revaccination was made compulsory in 2002 [41]. Another study of 779 samples obtained from St. Petersburg residents in 2013 [70] demonstrated that only 82.1 % of individuals were immune to rubella, indicating a weak effect of vaccination.

Data on the true prevalence of protective antibodies to mumps directly correlate with immunization coverage rates. Thus, according to State Research Center of Virology and Biotechnology VECTOR (Novosibirsk, Russia [71]), by the year 2006 a total of 90–95 % of the population had been vaccinated against mumps. The authors of [72] report that 97 % of 60 children examined in Novosibirsk at that time had antibodies to mumps. The study [73] carried out in Moscow in 904 individuals of different age revealed the presence of protective antibodies in 93 % of the participants.

In another study of post-vaccination immunity against hepatitis B conducted in 2013 in 970 residents of St. Petersburg, 55 % of the population were shown to have antibodies to HBsAg [74]. Interestingly, similar studies that used samples obtained before national hepatitis B immunization programs were launched (2001 [41]) yielded lower estimates of herd immunity. Thus, [75, 76] demonstrate that only 19–52 % of indigenous peoples of Siberia (sample sizes ranged from 194 to 657 individuals, samples were collected between 1993 and

2002) have antibodies to HBsAg. Similar data were obtained in another study [77] conducted in 302 Moscow residents in 2011–2013: antibodies to HBsAg were found in 20.9 % of the participants. Considering that mean age of the participants was 45.5 ± 8.6 years, one could assume that unvaccinated individuals prevailed in the sample. Based on the comparison of these three works, an inference could be made that the national hepatitis B immunization program is effective, if the data were not coming from such diverse populations.

The aforementioned methods of studying herd immunity in Russia cannot yield reliable results, even if they are used in combination. There is a need for a new national system of serological surveillance. But before elaborating on the issue, let us have a look at how serological surveillance operates abroad.

International programs for seroepidemiological surveillance and their results

To estimate immunization coverage, information systems are being developed or already operating in the USA and Europe, recording all administrations of vaccines to individuals. According to the criteria set by the Centers for Disease Control and Prevention [78], such systems:

– operate at the population level and are defined as computerized confidential databases that keep track of all vaccines and their doses administered by authorized providers to the residents of the region;

– promptly provide consolidated records of patient's immunization history;

– aggregate vaccination data at the population level for further use in epidemiological surveillance and public health control, or for estimating immunization coverage and incidence of vaccine-preventable infections.

In 2016 such systems were operating at the national level in 10 European countries (Germany, Sweden, Netherlands, Norway, and others); 5 countries had more than one subnational surveillance system (UK, Austria, etc.); 6 countries were launching pilot serosurveillance projects (France, Bulgaria, etc.) [79]. The global immunization plan for 2015–2020 describe these systems as "the integral part of a well-functioning health system" [80]. But although they ensure an accurate assessment of vaccination effectiveness, they fail to estimate the actual herd immunity, because administration of a vaccine does not necessarily lead to a successful immunization. Discrepancy between documented and actual vaccinations, imperfect tools for vaccine count and a few other factors promote the need for subnational and national projects aimed to study vaccine-induced immunity at the population level by collecting information (demographics, life histories, vaccination histories, etc.) and biosamples (blood). The samples are stored in biobanks [81] and used for laboratory-based seroepidemiological studies. The examples of national programs for serosurveillance are given below.

One of the vastest nation-wide studies of population immunity was conducted in the Netherlands and included two stages: the first referred to as PIENTER 1 took place in 1995–1996, and the second (PIENTER 2) — in 2006–2007; the project PIENTER 3 is ongoing. In the first stage 9,948 samples of blood serum were collected and tested for the presence of antibodies against 7 infections included in the national immunization schedule at that time, namely diphtheria, tetanus, measles, mumps, rubella and haemophilus influenzae type B [82, 83]. Samples were also tested for serological markers of hepatitis A, B and C (to detect chronic cases) and toxoplasmosis [84–86]. Data obtained in the course of PIENTER 1 were used to revise the national immunization plan; in the course of PIENTER 2 the list of surveyed infections was expanded to include meningococcal infection, human papillomavirus and varicella. In the second stage 6,386 samples were tested for the presence of 19 marker antibodies (for some pathogens more than one marker was proposed) [5, 87]. Both PIENTER 1 and PIENTER 2 were vigorously discussed in academic journals and national media, contributing to country's authority in the field of epidemiology.

In 2006 another study was conducted in Belgium aimed to estimate the prevalence of markers of 5 vaccine-preventable infections (measles, mumps, rubella, diphtheria and tetanus) in 3,974 samples. The study identified a few age groups with low immunity; additional immunization was recommended for these groups [88]. The data were confirmed by and supplemented with findings of Belgian researchers who also studied pertussis markers in 1,500 samples in 2012 [89, 20].

Between 2003 and 2006, another research study was carried out in Germany: 13,900 samples of blood were collected and tested for the presence of antibodies to measles, mumps and rubella. Initially, the samples were intended to be used in other public health programs [90]. So far, similar projects have been launched in China, Japan, USA, Italy, and other countries, signaling the significance of serosurveys. The summary of national surveillance programs is given in Table 1.

Importantly, serological studies provide a possibility to update herd immunity statistics in various social groups and regions, revise immunization schedules, estimate the effectiveness of immunization programs and the amount of vaccines and diagnostic assays required for their implementation.

Routine vaccination ensures sufficient resistance to specific pathogens but does not guarantee complete control over infection outbreaks, calling for additional programs that would cover particular age groups or regions at risk. Incident cases of infection in a susceptible population may indicate the need for vaccination, but it might be too late for the latter once an epidemic breaks out. It is easy to lose control over the situation if the infection has long been "forgotten", which does not mean that stable herd immunity has been fully established. Epidemiological modeling based on serological surveillance is a more reliable analytical tool [105]. For example, in response to a diphtheria outbreak in Eastern Europe, a serological survey was conducted revealing the lack of protective titers against the anatoxin, and an additional course of vaccination was proposed for the elderly population [106].

Serological surveillance has been helping to keep measles at bay in many countries, as it can identify the optimal vaccination age for susceptible groups of population. In the UK in the mid-1990s serological surveillance and mathematic modeling used in combination predicted a measles epidemic [107, 108]. Subsequently, a vaccination campaign was launched covering over 92 % of children aged 5 to 16 years. Further surveillance revealed that although the campaign was, on the whole, successful, it did not reduce susceptibility to measles in preschool children. This evidence was sufficient to prove the necessity of second dose administration of the MMR-vaccine (Measles/Mumps/Rubella) as part of immunization routine. A similar situation was observed in Australia [110, 111]. Those examples prove the importance of serological surveillance for maintaining herd immunity using vaccines with well-known properties.

Examples of regular and timely revisions of national immunization schedules inspired by seroepidemiological studies in the Netherlands indicate that serosurveillance has become an irreplaceable analytical tool [5]. Based on

PIENTER 1 results, routine vaccination was introduced against pneumococcal infection, the hepatitis B immunization schedule was revised, the Infanrix-IPV + Hib vaccine was substituted with Pedicacel, and a recombinant vaccine was proposed instead of previously used separate vaccines (DT-IPV and aP at the age of 4) [112]. All changes to immunization schedules were assessed in the course of PIENTER 2. During PIENTER 2, vaccines against meningococcal infection were introduced to be administered at 14 months and 19 years (in parallel with MMR), and a new vaccine against human papillomavirus was proposed for girls at 12 years [5]. Effectiveness of new vaccines and duration of vaccine-induced immunity can be estimated by further serosurveillance. Studies of genotype composition of circulating strains and their serotypes can also stimulate development of new effective vaccines [113–115].

Laboratory techniques for studying herd immunity

Screening tests are an essential component of serological surveillance, contributing to our understanding of herd immunity. According to [68], the following diagnostic techniques are employed by serological studies: the indirect hemagglutination test (IHAT) for the detection of antibodies to measles virus, diphtheria and tetanus anatoxins; the agglutination test (AT) for the detection of pertussis agglutinins; the enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to measles, rubella, mumps, hepatitis B and pertussis; the tissue-culture virus-neutralization test for the detection of antibodies to polio. ELISA is a very common method for quantitative and qualitative estimation of IgG antibodies in human blood serum. However, it may not be the best option when a sample needs to be tested for several different infections or when the number of samples is too big, because each marker is analyzed in a separate run. Therefore, large-scale serological studies of vaccine effectiveness employ multiplex immunoassays (MIA) capable of determining immunoglobulin antibodies to several antigens in parallel. Advantageously, MIA can be performed on very small serum samples (5 μL) [116], making this assay type convenient for underage patients. Multiplex assays are also very flexible as they can be expanded to incorporate additional antigens [117].

There are a few commercial platforms for multiplexing. Although the techniques and reagents for sample preparation and marker detection employed by these systems are different, all of them utilize polystyrene microsphere beads as solid phase. One of the most popular diagnostic platforms is xMAP (Multiple Analyte Profiling). Its key component are polystyrene beads internally labeled with different ratios of 2 to 3 spectrally distinct fluorophores thus generating 50 to 500 unique bead regions and subsequently combining the beads into a multiplex assay [118].

Importantly, the majority of multiplex assays for serosurveillance are in-house manufactured and optimized for specific tasks. Designed to quantify blood serum IgG antibodies to at least one pathogen, they have a capacity to simultaneously work with up to 23 different antigens, such as pneumococcal capsular polysaccharides [119]. Some researchers propose their own multiplex platforms in order to compare their performance with standard immunoassays, evaluate their sensitivity, reproducibility and specificity. Others seek to design a good diagnostic tool for serological surveillance that would be capable of simultaneous detection of several infections. For example, the Belgian team has proposed an assay for measuring antibodies to diphtheria, tetanus and pertussis to estimate herd immunity of Belgium residents to these infections, compare the obtained data with previous serological findings and decide whether this novel platform can be used in large-scale research studies [20]. This assay incorporates 5 antigens: diphtheria, tetanus and pertussis toxins, filamentous hemagglutinin and pertactin, linked to the surface of magnetic polystyrene beads [118]. Its performance was tested using serum samples of Belgians aged 20–29.9 years; the assay was then validated using a different panel of 37 serum samples and compared with ELISA results. It was discovered that 26.4 % of the participants had low levels of antibodies to diphtheria toxin (below the protective threshold, < 0.1 IU/ml). Susceptibility to tetanus was observed in 8.6 % of cases. A good correlation was observed between the multiplex and monoplex assays [20]. The multiplex assay results also correlated well commercial ELISAs (Table 2). According to FDA (Food and Drug Administration, USA) and EMA (European Medicines Agencies, EC) guidelines, a validated assay for antibody quantification is expected to demonstrate precision of ≤ 20 % coefficient of variance [120]. Therefore, we conclude that results of an xMAP-based MIA are highly precise. Another important characteristic of a diagnostic serological test is the limit of detection (LOD) unique for each antigen in the multiplex. LOD established for the described pentaplex assay was sufficient for screening purposes (Table 2).

Another assay was proposed by Dutch researchers for the detection of antibodies to the same 5 antigens [121]. Using a small serum sample, the team attempted to assess how reliable their assay was for serological diagnosis. It proved to have a lower LLOQ (lower limit of quantitation) than ELISA: 0.00078 EU/ml vs. 2 AU/ml for the pertussis toxin, respectively, and 0.00006 IU/ml vs. 0.01 IU/ml for the diphtheria toxin, respectively [121]. But the correlation coefficient was quite high (Table 3).

Specificity of each monoplex component of a multiplex assay is often tested by conducting homologous and heterologous inhibition experiments [116, 119–121]. The serum sample containing high levels of IgG antibodies to all studied infections is divided into aliquots whose number corresponds to the number of antigens; then each aliquot is incubated with one of the antigens. After preincubation, a suspension of antigen-bead complexes containing all studied antigens is added to the serum. Specificity of each monoplex is inferred from the comparison with the unpreincubated control. It is believed that the acceptable limit of detection (observed during homologous inhibition test) is 80–120 % [120]. Specificity of a multiplex assay created by Dutch researchers [121] proved to be very high (Table 2). Its diagnostic characteristics were quite good, and throughput capacity was remarkable; detection was rapid, and the amount of samples and antigens required for the analysis was minor.

Another innovative triplex assay was created by American researchers for the detection of *Corynebacterium diphtheriae*, *Clostridium tetani* and *Haemophilus influenzae* type b [122]. The assay incorporates three antigens: tetanus and diphtheria toxoids and the capsular polysaccharide of *H. influenzae* conjugated to human serum albumin (HbO-HA). It was designed to measure immunogenicity of combined vaccines and was used to reveal that 92.6 % of the studied serum samples had protective levels of IgG antibodies to the tetanus toxoid (≥ 0.1 IU/ML), 80.2 % — to the diphtheria toxoid (≥ 0.1 IU/ml) and 39 % — to the capsular polysaccharide of *H. influenzae*. Reference measurements were performed with ELISA. The correlation between the two methods was strong (Table 2) both for pre- and postvaccination samples [123].

Of particular interest are multiplex assays for the detection of antibodies to *Streptococcus pneumoniae* (Table 2). As a rule, such assays incorporate a large variety of antigens ranging from 9 [117] to 23 serotypes of the capsular polysaccharide [119]. Reliable detection of pneumococcal polysaccharides is more complicated resulting in a few assay modifications. First, poly-L-lysin coating is used to ensure better coupling of polysaccharide molecules to the polystyrene beads surface,. Second, there is a need to exclude antibodies specific to soluble pneumococcal cell wall polysaccharides and polysaccharide C typical for non-capsular strains of *S. pneumoniae*, because they do not confer immunity against this pathogen. Therefore, solution used to dilute the serum should contain absorbents - soluble polysaccharides of the cell wall, including polysaccharide C and serotype 22F polysaccharide[117, 122]. The same modifications are introduced to reference ELISAs, improving specificity of the assay and revealing its actual sensitivity.

British researchers designed a multiplex assay for quantification of IgG antibodies to polysaccharides of 9 *S. pneumoniae* serotypes. The assay was validated using the standard reference serum 89-SF [117]. Then, the assay was run on the serum samples of individuals who had and had not been previously vaccinated against *S. pneumoniae*. ELISA was used as a reference method. The original multiplex showed the lack of interference between its monoplex components, was sufficiently specific and sensitive, as required by FDA and EMA: the coefficient of variance did not exceed 20 % (Table 2). Besides, the multiplex proved to be stable after being stored for 12 months at +4 °C, showing only a slight decrease in fluorescence intensity by 10–19 %. The assay showed good correlation with ELISA, therefore it might be a better option for monitoring herd immunity against *S. pneumoniae* since it is time-saving and capable of detecting antibodies to all antigens contained in the vaccine.

An assay for detecting IgG antibodies to polysaccharides of 13 pneumococcal serotypes was developed by a research team from the Netherlands [124]. The assay was developed to measure humoral immune response to a 7-valent conjugate vaccine (PCV-7). Blood serum samples were collected from PCV-7 prevaccinated infants at 2, 3 and 4 months of age and at 11 months of age after booster vaccination. A few nonvaccine polysaccharide serotypes were also incorporated into the assay, revealing elevated levels of IgG antibodies to nonvaccine serotype 6A, resulting from its cross-reactivity with serotype 6B. Geometric mean concentrations of antibodies measured by the original multiplex immunoassay were higher than those measured by standard ELISA, but the coefficient of correlation between the two assays was high (Table 2). Differences between ELISA and MIA results suggest that the established protective threshold of 0.35 μg/ml should be reconsidered for the use in MIA and probably analyzed for each serotype separately [124]. A similar study was conducted a few years earlier in the USA (Table 2) [122]. An immunoassay was designed to simultaneously detect antibodies to 14 pneumococcal polysaccharide serotypes. The assay was run on the serum samples obtained before and after vaccination. Specificity of the assay was tested by conducting homologous and heterologous inhibition tests and turned out to be high for all polysaccharide serotypes except for two closely related serotypes 9N and 9V. However, with some serotypes heterologous inhibition was as high as 50 %. Cross reactivity could not be avoided by preincubation of serum samples with pneumococcal polysaccharide C, but preadsorbtion with polysaccharide serotype 22F considerably reduced the level of heterologous inhibition. The results obtained with ELISA and MIA in this study were highly concordant.

The uniqueness of another assay designed to quantify IgG antibodies to polysaccharides of *S. pneumoniae* lies in its capacity to simultaneously detect immunoglobulins to 23 polysaccharide serotypes (Table 2) [119]. Apart from polysaccharide serotypes coupled to microsphere beads, the assay incorporated an internal control — microspheres coupled with cell wall polysaccharides of *S. pneumoniae* and the second control — microspheres coupled with nonvaccine serotype PnPS25 polysaccharide used to evaluate reproducibility of the results, which is critical for pre- and post-vaccination studies. The assay was validated using antipneumococcal standard reference serum 89S-2 (FDA) containing IgG to pneumococcal antigens extracted from the blood serum of individuals vaccinated with a 23-valent vaccine against *S. pneumoniae*. The ELISA and MIA results were highly correlated, while homologous inhibition was observed for all serotypes except for polysaccharide serotypes 2, 3 ,4, and 5 (no heterologous inhibitions was observed for these serotypes either) [119].

Routine immunization against viral infections such as measles, mumps, rubella and varicella is carried out in the majority of WHO member states. Multiplex assays are also available for measuring immune response to these pathogens. In the study [116] native purified antigens coupled with magnetic microspheres were used (Table 2). The assay was run on 70 samples of human blood serum of vaccinated and unvaccinated individuals. The researchers concluded that their xMAP-based assay was highly specific and sensitive. Besides, LLOQ was calculated for each analyte (Table 3) demonstrating that the x-MAP-based assay was more sensitive to the studied infections than ELISA.

Given their excellent diagnostic characteristics and cost-effectiveness, multiplex assays should be used in large-scale serological studies.

Promising methods for evaluation of immunological memory

Although immunological techniques are becoming increasingly common and provide a wealth of information for serological surveillance, there is growing evidence indicating the need for novel approaches [125]. Current techniques are unable to evaluate the cell-mediated immunity and cannot be tailored to identify infection markers in a particular cell population of interest. They can also simultaneously detect only a few markers. New approaches will have to account for the fundamental properties of immunological memory and molecular features of pathogens. Novel methods can lead to discovery of unknown properties of the immune system or new interpretations of the already known facts. Detection of specific antibodies is the primary method of assessing immunological memory described in the previous sections of the article. In this section we will speak about the limitations of this approach and briefly analyze the new methods employed by epidemiological studies.

Immunological memory is a dynamic parameter of the adaptive immunity. It changes throughout a person's life, influenced by infections, vaccinations, microbiota composition and lifestyle [125–128]. First, the ratio of native to immune memory cells decreases with age, resulting in deteriorating immunological memory characteristics [129–131]. The diversity of adaptive immunity receptors in infancy is similar across the population, but at older age differences become more visible mirroring the events that have occurred in person's immune system throughout life [127, 128]. Compositions

Table 1. Serological surveillance projects

Reference	National program/ project	Country	Infection	Problem/objective	Study duration	Method	Number of participants
[91]	National Epidemiological Surveillance of Vaccine-Preventable Disease (NESVPD)	Japan	Pertussis	In 2013 high prevalence of of antibodies to pertussis toxin was discovered in the adult population in Japan.	2013–2014	In-house ELISA	252
[92]	Well-Child-Care Program of Ambulatory Healthcare Services	UAE	Influenza A and B	Vaccination against flu is recommended for high-risk groups only. The objective of the program is to prove that nation-wide vaccination against influenza A and B is necessary for all children.	2014–2015	Commercial ELISA	294
[93]			Measles, mumps, rubella, pertussis, diphtheria, tetanus, polio, varicella, Hib-infection	To estimate prevalence of antibodies in children to 9 vaccine-preventable infections listed in the immunization schedule.			227
[94]	Independent serological study	Israel	Hepatitis A	To estimate seroprevalence of IgG in Israeli population unvaccinated against hepatitis A and vaccinated after this infection was included into the immunization schedule for infants.	1997–1998 and 2011	Commercial ELISA	1,883 — before vaccination 2,027 — 12 years after vaccination
[95]	DRC Demographic and Health Survey (DHS)	Congo	Rubella	Vaccination against rubella is not recommended by the immunization schedule in Congo. The study aims to estimate the burden of the infection.	2013–2014	Commercial ELISA	7 195
[96]	Healthy Life In an Urban Setting (HELIUS)	Netherlands	Human papillomavirus infection	To determine whether differences in the prevalence of antibodies to high risk type papillomavirus are significant between men and women of different ethnicities.	2011–2014	In-house MIA	4 637
[97]	The European Sero-Epidemiology Network (ESEN).	Italy	Pertussis	To measure seroprevalence of pertussis in adults residing in the countries with good vaccination coverage in the backdrop of increasing pertussis incidence.	1996–1997 and 2012–2013	ESEN-standardized ELISA	637 — in 2012–2013; 1,037 — in 1996–1997
[98]	The National Measles Case Based Surveillance System	Ethiopia	Rubella	To study seroprevalence of rubella in Ethiopia in order to decide on the inclusion of rubella vaccine into the national immunization schedule.	2009–2015	Commercial ELISA	17 066
[99]	Independent serological study	Nicaragua	Hepatitis A	Two-dose vaccination against hepatitis A is an effective but costly procedure in the endemic areas. To estimate effectiveness of single-dose vaccination.	2003–2012	Commercial ELISA	130
[100]	Independent serological study	Cambodia	Tetanus	Nation-wide serological study of immunity against tetanus in Cambodian women aged 15 to 39 years.	2012	In-house ELISA and MIA	2 150
[101]	PIENTER 2	Netherlands	Diphtheria	To evaluate the national immunization program against diphtheria; to compare its results with the previous project carried out 11 years ago.	2006–2007	In-house MIA	6,383 — after vaccination; 1,518 — without vaccination
[102]	Independent serological study under the aegis of Chinese CDC	China	Pertussis, diphtheria	To measure humoral immunity against diphtheria and pertussis in Beijing.	2012	Commercial ELISA	2 147
[103]	Independent serological study under the aegis of Chinese CDC	China	Hepatitis B	To measure humoral immunity against hepatitis B in a Chinese province in order to compare the effect of vaccination with the results obtained 12 years ago.	2012	Commercial ELISA	13 207
[104]	National Health and Nutrition Examination Survey (NHANES I and III)	USA	Varicella	To determine seroprevalence of varicella in the American population in 1999–2004.	1999–2004	ELISA (CDC protocol)	16 683

of cell populations, as well as cytokine and immunoglobulin profiles, are different between healthy people [125], shaped by genetic factors, age, sex, gut microbiota, vaccination, lifestyle, environment, season, and circadian rhythms. But little is known about the impact of these factors on the qualitative characteristics of immunity. There is an ongoing active search for markers that can link qualitative and quantitative characteristics of the immune system with the existing pathology or the risk of its development [125].

The article [21] calls for the establishment of a world serology bank and proposes to divide all pathogens into 4 groups. The first group includes acute immunizing antigenically stable pathogens (measles, rubella and smallpox). The second group contains acute immunizing antigenically variable pathogens (flu, invasive bacterial infections, dengue). The third group comprises infections for which antibodies are not protective; among those are tuberculosis, that induce different immune response depending on the infection stage; malaria that triggers production of several antibody types by infected red blood cells; and HIV. The fourth group is the largest and includes infections that do not induce sustained immune response or trigger production of specific antibodies that, however, do not

Randomization	Result
Three age groups: 4-7 years, 10-14 years, and 35 years, 84 individuals from 6 prefectures per group. Participants' personal data: sex, age, residential area and date of sample collection.	High seroprevalence of antibodies to pertussis toxin is not related to vaccination but results from the disease in adult life.
Age range: 1.9 – 12.5.years, median age: 4.1 years. Participants: unvaccinated children without acute or chronic conditions and regular medications.	Type A seronegative children – 77%; type B seronegative children – 59%. The majority are susceptible to the infection, therefore routine vaccination can be recommended for this age group.
Age range: 1.9–5.9 years. Participants: children vaccinated as recommended by the national immunization schedule, without acute or chronic conditions and regular medications.	30% to 60% of children had low serum levels of antibodies to pertussis, varicella and mumps. The national immunization program should be revised.
Serum samples provided by the bank were obtained from Israelis residing in all regions of the country. Personal information available: sex, age (0–1.5; 1,6–4; 5–13; 14–19; 20–24; 25–34; 35–44; 45–54; 55–64; 65+ years), place of residence, date of blood collection, ethnicity (Jew or Arab).	Age-adjusted levels of HAV-antibodies increased from 47% to 67% and from 83% to 88% in Jews and Arabs, respectively. Low incidence rates of hepatitis A is a result of vaccination against this infection
The study was conducted in infants between 0.5 and 4.9 years of age. Stratification was based on age, sex, number of siblings, maternal age and education, residence. Additional personal information available: vaccination history.	33% of children were IgG-positive for rubella.
Randomly selected 4 ,637 not vaccinated against HPV male and female residents of Amsterdam aged 18 to 44 years stratified by ethnicity (Dutch, Surinamese, Ghanaian, Moroccan and Turkish). The authors selected 20 participants per life year, in the age range from 18 to 44 years (=27 life years).	Unlike men, seroprevalence of high risk type HPV was reliably different between women of different ethnicities.
Results of the study of 637 serum samples obtained from different age groups (20–29, 30–39 and 60+ years) in 2012–2013 were compared to the results of the study of 1, 037 serum samples collected between 1996 and 1997.	Increased circulation of pertussis causative agent in the Italian adult population requires additional preventive strategies.
Serum samples were obtained from patients with suspicion of measles who were serologically negative for this infection. Randomization was based on sex, age (< 1 year, 1–4, 5–9, 10–14, > 14 years) and residence.	Rubella is highly endemic in Ethiopia and is highly incident in children <10 years of age. Rubella Surveillance System is necessary to decide on the inclusion of rubella vaccine into the national immunization schedule.
The study included children aged 1.7 – 17 years. Children were examined regularly during 7.5 years after single-dose vaccination against hepatitis A. Data available: demographic and socio-economic factors.	A single dose of HAV vaccine is sufficient for establishing long-term immunological memory in children from areas with highly endemic hepatitis A.
The study was conducted in women aged 15 to 39 years, stratified into age groups with a 5-year interval. The country was divided into 5 regional strata. Participants were randomly selected from 611 clusters in these regions.	Seroprevalence of tetanus in the studied population was 88%.
From each of 5 regions of the country, 8 municipalities were randomly selected with a probability proportional to their size. From each of 40 municipalities 380 to 500 individuals were selected and stratified by age (0, 1–4, 5–9, 10–14, ..., 75–79 years).	In spite of good vaccination coverage in general, the risk of diphtheria is high in some geographically clustered communities whose residents are vaccine-hesitant for religious reasons.
Age: from 3 months to 74 years. Stratification by sex, residence (locals or migrants), age (0–1, > 40 years, a 5-year interval for 1-40 years). Additional personal information available: vaccination history.	50%–70% of adult population of Beijing have no protective antibodies against diphtheria; the rate of antibodies to pertussis was <25% in all age groups indicating a potential risk of the infection spread.
Age: from 1 to 14 years (< 5, 5–9 and 10–14 years). Stratification by economic development (high, middle and low), 10 districts selected from each strata.	Prevalence of HBsAg and anti-HBc was 0.8 and 2.6%, respectively, in older groups, while in participants aged < 0.5 years it was 0.5%. The number of HBsAg-positive children decreased significantly due to the vaccination program started 20 years ago.
Age: 6–49 years. Stratification by age, sex, poverty index, marital status and place of birth.	With age, seroprevalence of varicella increased in children and was uniformly high in the adult population of the USA in 1999-2004.

confer immunity against this infection in the future. This group includes intestinal infections and human papillomavirus.

The first group induces sustained humoral response leading to production of protective antibodies. Detected in the sample, these antibodies reliably indicate a past infection or vaccination. With the second group, the presence of antibodies may confer protection against future infection; the antibodies can also be used in vaccine development and pandemic modeling. The existing serological assays can measure the level of resistance to both groups of infections. But they fail to identify whether immunity was induced by vaccination or naturally by a disease [21, 132]. For example, the presence of anti-HBsAg and anti-HBcore antibodies may indicate hepatitis B in the past, while the presence of only anti-HBsAg antibodies indicates that the person has been vaccinated [133]. Some infections may be detected using special immunological markers added to a vaccine [134]. The recombinant vaccine against tuberculosis GamTBvac developed by the authors of this article makes use of the response to the dextran-binding domain participating in deposition onto the dextrane adjuvant [135].

Antibodies produced in response to infections included in the third and fourth groups are not protective against future

invasions of the same pathogens. However, they indicate a current or past infection. Therefore, in the absence of reliable markers of established immunity against these groups of pathogens, serological findings can be used to assess vaccination coverage in the population, given that vaccination induces a long-lasting humoral response [21]. Discovery of trustworthy markers of induced immunity would be beneficial for epidemiological surveillance and could inspire development of novel effective vaccines. Vaccines against pulmonary TB, malaria and HIV are still nonexistent partly because no reliable markers of immune protection have been discovered so far. However, there are known cases of immunologically mediated resistance to infections belonging to groups 3 and 4. Here the absence of protective humoral response is explained by the crucial role of cell-mediated immunity [21, 136].

Cell-mediated immunity can be measured using the Interferon Gamma Release Assay (IGRA). This method is based on measuring the level of interferon-gamma (IFN-g) production by lymphocytes of whole blood or the PBMC-fraction (subpopulation of peripheral blood mononuclear cells) exposed to antigens [137]. IFNg levels are determined by ELISA. This approach is widely used to confirm TB diagnosis and detect other infections caused by intracellular pathogens (tularemia, leishmaniasis, cytomegalovirus infection) [138]. Unfortunately, the assay only indicates the presence of infection but does not detect any markers of immunity against it.

A search for the markers of immunity against infection can be aided by flow cytometry and its modification including cell sorting. This method allows analyzing single cells using fluorescently labeled antibodies and cell compartment markers by measuring up to 30 parameters simultaneously in hundreds of thousands and millions of cells [139]. This analysis yields a wealth of reliable information about differentiation and properties of cell subpopulations. The method can be used in routine epidemiological surveillance if the need should arise to search for immunity or sensitivity markers in particular cell populations. Developers should make the protocol for this method as simple as possible. There may be barriers hampering its introduction into routine practice, such as strict requirements for working with live cells. Using cell sorting by modified cytometers,

Table 2. Analytical characteristics of multiplex assays for serological studies

Reference	Country	Technology	Pathogens	Number of antigens	Serum sample size, n	Reproducibility	Limit of detection	Correlation with ELISA, R^2
[116]	Netherlands	xMAP Luminex	Measles virus, mumps virus, varicella zoster virus	4: native purified antigens	70	CV* for intra-assay variation within plate 5%–14% CV for intra-assay variation between plates 12%–16%	0.00024 — for measles virus, 0.053 — for mumps virus, 0.00129 — for rubella virus, 0.00024 — for varicella virus	0.953–0.983
[20]	Belgium	xMAP Luminex	*Bordetella pertussis, Corynebacterium diphtheriae, Clostridium tetani*	5: diphtheria, tetanus and pertussis toxins, filamentous hemagglutinin and pertactin	670	CV* for intra-assay variation within plate 0.44%–2.47% CV for intra-assay variation between plates 2.11%–2.67%	0.00031 — for DT*, 0.00035 — for TT*, 0.012 — for PT*, 0.032 — for FHA*, 0.2 — for Prn* (all measured in mAU/ml)	0.89–0.98
[122]	USA	xMAP Luminex	*Streptococcus pneumoniae*	14 polysaccharide serotypes of S. pneumoniae: 1, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 12F, 14, 18C, 19F, and 23F	50	Unpublished	Unpublished	0.85–0.95
[121]	Netherlands	xMAP Luminex	*Bordetella pertussis, Corynebacterium diphtheriae, Clostridium tetani*	5: diphtheria, tetanus and pertussis toxins, filamentous hemagglutinin and pertactin	28–70	CV for intra-assay variation within plate 7%–9% CV for intra-assay variation between plates 10%–13%,	0.00002 — for DT*, 0.00001 — for TT*, 0.26 — for PT*,0.10 — for FHA*, 0.22 — for Prn* (all measured in mAU/ml)	0.948–0.984
[119]	USA	xMAP Luminex	*Streptococcus pneumoniae*	23 polysaccharide serotypes of S. pneumoniae:1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F	Standard reference serum 89S-2	N/A	N/A	0.90 for serotypes 1, 4, 5, 6B, 9V, 14, 16C, 19F and 23F
[124]	Netherlands	BioPlex 100 (Bio-Rad)	*Streptococcus pneumoniae*	13 polysaccharide serotypes of S. pneumoniae: 1, 3, 4, 5,6A, 6B, 7F,9V, 14, 18C, 19A, 19F, 23F	188	N/A	N/A	N/A
[117]	Great Britain	BioPlex (Bio-Rad)	*Streptococcus pneumoniae*	9 polysaccharide serotypes of S. pneumoniae: 1, 4, 5, 6B, 9V, 14, 18C, 19F, 23F	120	CV* for intra-assay variation within plate 5.2%–8.34% CV for intra-assay variation between plates 12.1%–19.2%	32.3–109.7 pg/ml	0.91–0.96
[123]	USA	xMAP Luminex	*Corynebacterium diphtheriae, Clostridium tetani, Haemophilus influenzae тип b*	3: diphtheria toxoids, capsular polysaccharide of *Haemophilus influenzae*, conjugated with human serum albumin (HbO-HA)	81	N/A	N/A	N/A

Note. CV — coefficient of variance, DT — diphtheria toxin, TT — tetanus toxin, PT — pertussis toxin, FHA — filamentous hemagglutinin, Prn — pertactin.

targeted populations of cells can be separated and tested for the presence of immunity markers by different techniques. Sorted cells can be frozen and stored in the biobank until use.

Another promising method is based on estimating the diversity of antibody producing T-lymphocyte [128] and B-lymphocyte receptors by next-generation sequencing (NGS). Diversity of receptors in cell subpopulations provides a wealth of information about patient's immunological memory, including his/her medical history and the risks of possible infections in the future. State-of-the-art techniques can show what types of antibodies are produced by a particular person and what linear epitopes his/her immunological memory recognizes. Unfortunately, there is no way to identify specificity of all receptors in total. Advances in bioinformatics, cybernetics, artificial intelligence, machine learning, and large-scale studies initiated to investigate correlations between a phenotype and the variability of adaptive immunity receptor loci may provide the answer. Among the disadvantages of NGS are labor-intensive DNA-library preparations, high costs of the analysis, expensive equipment, and the need for highly skilled professionals.

Information obtained with the abovementioned techniques can be of great importance for fundamental immunology. If discovered, novel immunity markers will be adapted for use in simple diagnostic procedures, including multiplex immunoassays [140], IGRA-tests, polymerase chain reaction assays, flow cytometry [139] or mass spectrometry [141].

A proposal for a system of serological surveillance in Russia, expenses and economic rationale

Based on the international expertise and the knowledge of methods for studying herd immunity, we hereby propose a system for serological surveillance in Russia according to the principles mentioned in the first section of this article.

Surveillance should be performed by the Ministry of Healthcare of the Russian Federation, Rospotrebnadzor or another authorized agency capable of providing administrative resources for the interaction with regional healthcare facilities in order to facilitate sample collection. The following hierarchy is proposed.

1. Sample collection. Regional healthcare ministries should be invited to participate in sample collection, since it is in their best interest to have access to reliable epidemiological data; local healthcare facilities should be engaged considering that samples should be obtained from healthy individuals. At this stage participants are recruited, questionnaires filled out, samples collected, processed (serum extraction) and transported to the biobank.

2. Registration, certification and storage of samples in a specialized national serum biobank [23]. The biobank is central [142] to the surveillance system, because it a) accumulates collected samples, aliquotes them and stores for a long period of time under standard conditions; b) automatically processes information about the samples, including the date and location of sample collection, donor personal records, and study results, which considerably reduces the probability of mistakes during testing or result processing, increases reliability of the obtained data, and allows automated receiving and processing of serological statistics.

3. Laboratory screening of the obtained samples for the presence of serological markers using multiplex ELISAs for simultaneous quantification of tens of different markers in a sample, which will reduce costs and time required for the procedure. Authorized laboratories involved are expected to have technical capacities to work with blood samples, which may contain group III and IV biological agents, and to perform culture-independent diagnostic tests for group II biological agents [143, 144].

4. Analytical center for data processing and epidemiological prediction that will also present exhaustive information to public healthcare or other agencies.

According to the international serological surveillance projects, revision of immunization programs should rely on the data obtained from 0.02–0.05 % of the country's population [5, 20, 87, 88, 90], which is 30–75 thousands of seemingly healthy people in Russia. Study participants should give their informed consent and complete the questionnaire form providing information on their age, sex, socio-economic status, residence, previous vaccinations, chronic infectious diseases, and risk factors. Participants should be motivated by a modest payment and a detailed feedback on their immunity status.

All samples should be stratified into cohorts of at least 500 individuals based on specific epidemiological parameters. Cohorts over 500 individuals ensure accurate estimation (confidence interval) of infection marker carriers proportion with ± 5 % precision at the population level for the seroprevalence of 40–60 %, and with ± 2.5 % precision at the population level for the seroprevalence of 90 % to 100 % ($p < 0.05$) [145], which is sufficient for the analysis of herd immunity [88]. Stratification should be primarily based on age. Vaccination schedules and age-related changes of the adaptive immunity [146, 147], as well as data obtained in the course of similar projects abroad [148] suggest that stratification into groups should be based on vaccination ages recommended by the national immunization schedule [30]: children before 1, 2, 3, 4, 5, 6, 12, 15 and 18 months of age; before 2 years of age and older until the age of 11 years with 1-year interval; from 11 until 35 years of age with 3-year intervals; from 35 years of age with 5-year intervals (there should be no less than 35 age groups in total each including over 500 individuals). Because mass sample collection in infants below 12 months of age is hampered by technical and ethical difficulties, in the pilot stage of the project children can be stratified based on the established decreed ages specified in Form 6 [28]: before 12 months, before 2 years, with 1-year intervals until the age of 18. This stratification approach will help to estimate the actual number of immune members of the population and to study effectiveness of immunization programs in all age groups.

Of importance is territorial and socio-economic stratification. We believe that the system of healthcare in Russia provides equally effectives services (at least, as far as immunization programs go) for all Russian residents regardless of their area of residence or income. However, this supposition can only be confirmed or disproved by serological surveillance. Besides it should be remembered that endemicity of infection depends on the climate zone and the economic status of the region, thereby affecting local herd immunity. Stratification proposed by the Russian Longitudinal Monitoring Survey (RLMS) conducted between 1992 and 1998 [149] appears to be quite adequate for our purposes. The entire territory of Russia was divided into 10 geographical and 10 life quality strata. To ensure

Table 3. Correlation between MIA and ELISA results (Smits et al., [116])

Virus	MIA LLOQ, mIU/ml	ELISA LLOQ, mIU/ml
Measles	0.72	20
Rubella	3.86	3 000
Varicella	0.71	660
Mumps	160	4 000

representativity of the sample (individuals registered with a local healthcare facility and households), the method proposed in the ESSE-RF study (2012–2014) [150] was used, similar to that of NHANES (National Health and Nutrition Examination Survey [151]). The authors of these works claim that their method is well-established and suitable for big and diverse populations.

The range of serological markers should be defined. We recommend including in the surveillance all vaccine-preventable infections listed in the national immunization schedule. This list contains 23 infections. Because of seasonal mutations, a separate method should be elaborated for flu strains [152, 153] and tuberculosis, because the presence of IgG antibodies in blood does not indicate protection against these infections [135]. We also recommend using ELISA as a diagnostic tool.

Ideally, cross-sectional studies [25] of herd immunity at a particular time point at the population level should be conducted on a regular basis, once every 6 or 7 years, because this interval is sufficient to establish a stable immunity to vaccine-preventable infections or infections in general [31, 146, 154] and indicates succession of “immune generations”.

According to our estimates, one round of serological surveillance in 10 pilot regions (30,000 samples, 30 antibodies) will cost 300 million rubles, including 62 million rubles for sample collection (payments to participants and medical personnel involved, expenses on consumables and paper work); 23 million rubles for modernization of regional centers for sample collection and preparation; 16 million rubles for transportation; 99 million rubles for multiplex immunoassays; 100 million rubles for expenses on analytical centers (biobank stewardship, equipment, paychecks, administrative costs). We believe that long-term results of serological surveillance will justify expenditures of the state.

Economic effect from serological surveillance can be expected to emerge after additional vaccination of susceptible population groups as the economic burden caused by consequences of vaccine-preventable diseases will decrease. A method to assess this type of burden was proposed by the experts of the Central Research Institute of Epidemiology [155, 156]. According to [34, 50], the state loses a total of 6 billion rubles as a result of infections that can be prevented by vaccination (pertussis, measles, mumps, tetanus, diphtheria, rubella, acute hepatitis B, hepatitis A, brucellosis, tularemia, typhoid fever, rabies, hemorrhagic fevers, including tick-borne encephalitis and yellow fever). Strong herd immunity against these infections aided by additional vaccination of susceptible population groups will be economically beneficial. In this light, 300 million rubles appear to be a reasonable cost, even if additional vaccinations will be necessary. The ratio of expenses to the long-term beneficial economic effect is estimated between 1 : 5.7 and 1 : 14.4 [157], rendering vaccination programs economically justifiable.

CONCLUSION

Increasingly intricate immunization schedules, migration, ageing, and vaccine hesitancy call for the need to introduce a system for population-wide serological surveillance capable of estimating herd immunity against infections and identify susceptible population groups. This system will help to update statistics on herd immunity in different social groups and regions, revise the national immunization schedule, calculate the amount of vaccines, diagnostic tools and efforts required for protecting the nation against infections, as described in [23].

Current methods applied in the studies of herd immunity are not comprehensive and cannot be used as reliable prognostic tools: data on vaccination coverage and infection incidence are often unrepresentative of the entire country’s population and the serological studies are too few, limited to a certain area and contradict the basic principles of serosurveillance (adequate coverage, continuity and homogeneity of data).

The effective system for serological surveillance should include collection of samples representative of the studied population, establishing biobanks of serum, DNA, RNA and cell samples, development of screening tests for the immunological memory qualitative assessment and epidemiological modeling based on the data on herd immunity. A surveillance system is crucial for developing a wise vaccination strategy and ensuring protection against biological threats guaranteed by the Constitution of the Russian Federation.

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IMMUNOLOGICAL MEMORY FORMED IN RESPONSE TO ADMINISTRATION OF GamTBvac RECOMBINANT TUBERCULOSIS VACCINE CANDIDATE: CLINICAL TRIALS IN HEALTHY VOLUNTEERS

Kleymenov DA[1] ✉, Mazunina EP[1], Lunin VG[1], Koptev EYu[1], Manuilov VA[1,2], Gushchin VA[1,3], Tkachuk AP[1]

[1] Laboratory of Translational Biomedicine,
N. F. Gamaleya Federal Research Centre for Epidemiology and Microbiology, Moscow, Russia

[2] Institute of Molecular Medicine,
I. M. Sechenov First Moscow State Medical University, Moscow, Russia

[3] Department of Virology, Faculty of Biology,
Lomonosov Moscow State University, Moscow, Russia

So far BCG, a live attenuated *Mycobacterium bovis* strain remains the only available vaccine for tuberculosis prevention and control. Although BCG is effective against miliary tuberculosis and tuberculous meningitis in children, it barely protects adults and adolescents from the pulmonary form of the disease or reactivation of the latent infection. Still, its effectiveness can be increased by using recombinant booster vaccines containing both *M. bovis* and *M. tuberculosis* antigens. This article reports preliminary data on the safety and immunogenicity of a recombinant vaccine candidate, GamTBvac, developed for tuberculosis prevention. Its immunogenicity was studied in 12 volunteers. Over the course of 20 weeks following GamTBvac administration, we measured cell-mediated and humoral immune responses using interferon-gamma release assays and multiplex xMAP-based immunoassays. On day 140 after the first administration of the vaccine, 10 participants of the study (83 %) still showed a positive cellular response to all antigens contained in the vaccine. Both sense antigens CFP10 and ESAT6 induced production of IgG antibodies between days 98 and 140 of the observation. The Ag85 antigen induced a relatively weak humoral response. On the whole, the recombinant GamTBvac is safe and activates cell-mediated and humoral components of the adaptive immunity, forming immunological memory.

Keywords: immunological memory, humoral immunity, cell-mediated immunity, tuberculosis, BCG, *M. bovis*, *M. tuberculosis*, IGRA, suspension immunoassay, ESAT6, CFP10, Ag85

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✉ **Correspondence should be addressed:** Denis Kleymenov
ul. Gamalei, d. 18, Moscow, Russia, 123098; 10000let@rambler.ru

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ИММУНОЛОГИЧЕСКАЯ ПАМЯТЬ, ФОРМИРУЕМАЯ В ОТВЕТ НА ВАКЦИНАЦИЮ ПРОТИВОТУБЕРКУЛЕЗНОЙ РЕКОМБИНАНТНОЙ ВАКЦИНОЙ «ГамТБвак»: КЛИНИЧЕСКИЕ ИССЛЕДОВАНИЯ ВАКЦИНЫ НА ЗДОРОВЫХ ДОБРОВОЛЬЦАХ

Д. А. Клейменов[1] ✉, Е. П. Мазунина[1], В. Г. Лунин[1], Е. Ю. Коптев[1], В. А. Мануйлов[1,2], В. А. Гущин[1,3], А. П. Ткачук[1]

[1] Лаборатория трансляционной биомедицины,
Национальный исследовательский центр эпидемиологии и микробиологии имени почетного академика Н. Ф. Гамалеи, Москва

[2] Институт молекулярной медицины,
Первый Московский государственный медицинский университет имени И. М. Сеченова (Сеченовский Университет), Москва

[3] Кафедра вирусологии, биологический факультет,
Московский государственный университет имени М. В. Ломоносова, Москва

В настоящее время единственной применяемой в мире вакциной против туберкулеза является БЦЖ — живой аттенуированный штамм *Mycobacterium bovis*. Она защищает детей от милиарного туберкулеза и туберкулезного менингита, но не уберегает взрослых от легочного туберкулеза и реактивации латентной формы инфекции. Для повышения эффективности вакцины БЦЖ у взрослых и подростков разрабатываются рекомбинантные бустерные вакцины, несущие антигены как *M. bovis*, так и *M. tuberculosis*. В статье приводятся первые данные о безопасности и иммуногенности кандидатной рекомбинантной вакцины для профилактики туберкулеза «ГамТБвак». Изучали иммуногенность препарата на 12 добровольцах. Оценку проводили по изменению у испытуемых параметров клеточного и гуморального иммунитета (методами IGRA-тест и мультиплексный иммунологический xMAP анализ соответственно) в течение 20 нед. после введения препарата. На 140-й день с момента первой вакцинации у 10 (83 %) из 12 иммунизированных «ГамТБвак» добровольцев сохранялся положительный клеточный ответ на все антигены, входящие в состав вакцины, по сравнению с уровнем до вакцинации. Оба смысловых антигена CFP10 и ESAT6 индуцировали достоверную выработку IgG антител с 98-го и 140-го дней наблюдения соответственно. Антиген Ag85A вызывал сравнительно низкий гуморальный ответ. В целом, изучаемая рекомбинантная вакцина «ГамТБвак» обладает необходимым уровнем безопасности и активирует клеточное и гуморальное звенья адаптивного иммунитета, формирует клеточную память.

Ключевые слова: иммунологическая память, гуморальный иммунитет, клеточный иммунитет, туберкулез, БЦЖ, *M. bovis*, *M. tuberculosis*, IGRA, суспензионный иммунологический анализ, ESAT6, CFP10, Ag85

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✉ **Для корреспонденции:** Клейменов Денис Александрович
ул. Гамалеи, д. 18, г. Москва, 123098; 10000let@rambler.ru

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According to the estimates in [1], tuberculosis has killed over 1 billion people in the past 200 years — more than smallpox, plague, malaria, influenza, cholera and AIDS combined. By the end of the 19th century, one in every five deaths was tuberculosis-related [1]. The situation improved after the BCG (Bacillus Calmette–Guérin) vaccine was introduced in the 1920s. It was hoped that tuberculosis would be completely eradicated, given a drastic decrease in incidence rates in Europe and the US [2]. But that did not happen, largely due to the properties of the BCG vaccine itself. For example, it was discovered that although BCG does protect children from miliary tuberculosis and tuberculous meningitis, it still offers limited protection from the aerosol infection to teenagers and adults. Besides, effectiveness of vaccination varies across regions, from high in Europe and North America to almost negligible in the equatorial areas [2]. Thereby, although tuberculosis is curable and preventable, it still remains one of the top three causes of death from infection. In 2015, 10.4 million new cases of active TB were reported, and at least 1.8 million people died from this disease [3].

One of the troubling aspects of tuberculosis is the increasing incidence of its multidrug-resistant forms. In 2015 about half a million new cases of multidrug-resistant tuberculosis (MDR TB) were reported. Unfortunately, therapies for MDR TB are very expensive (over $ 10,000 per treatment course), with successful treatment outcomes of only 50 %. Experts estimate, that today about 50 million people all over the world have latent MDR TB. The probability of its reactivation throughout life is over 10 % [4]. MDR tuberculosis is a pressing issue in Russia. A joint contribution of India, China and Russia to the global incidence rate is 45 % [3]. In Russia alone incidence remains as high as 115 cases per 100,000 people, going up to 160 cases in some regions [5].

WHO's global plan is to reduce TB incidence and mortality by 90 % and 95 %, respectively, by 2035 [3]. This ambitious goal cannot be achieved without novel effective vaccines. Recombinant vaccines containing *Mycobacterium tuberculosis* antigens, some of which are present in BCG, make up the large proportion of all vaccines developed today [6]. These vaccines are boosters aimed to reinforce the immune response previously induced by BCG and are not intended for primary immunization of neonates, which determines their antigen composition [7]. One of such vaccines is the recombinant GamTBvac containing two mycobacterial antigen fusions (Ag85A and ESAT6-CFP10) with dextran-binding domain (DBD) immobilized on dextran. The adjuvant is represented by DEAE-dextran core and CpG oligonucleotides (TLR9 antagonist). Rationale for vaccine composition and formulation can be found in our previous work [8].

Preclinical studies have demonstrated strong immunogenicity and efficacy of GamTBvac in mice and guinea pigs [8]. GamTBvac has been shown to confer protection against the H37Rv strain of *M. tuberculosis* under aerosol and intravenous challenges. As anticipated, GamTBvac was particularly effective when used as a booster in animals who received a BCG prime. Following the successful completion of the preclinical trial, we obtained an approval from the Ministry of Healthcare of the Russian Federation (authorization ID 179 dated April 10, 2015) to initiate a clinical study (CS) of GamTBvac's safety and immunogenicity in BCG-vaccinated healthy volunteers. The protocol of the study is available in the international database NIH [9]. Below we report the first results of this study.

METHODS

Clinical study design and protocol

The clinical study was conducted in compliance with the laws of the Russian Federation and in accordance with domestic and international regulations and ethical standards [10–13]. Our phase I/IIA study of immunogenicity and safety recruited 60 healthy BCG-vaccinated male and female volunteers aged 18 to 49 years. The study was designed to have three stages (Fig. 1). Among the exclusion criteria at screening (besides ongoing drug therapies and acute conditions) was a positive QuantiFERON-TB Gold (Qiagen, USA) test for latent tuberculosis [14, 15]. In the first stage, we studied safety of a single GamTBvac dose in 24 volunteers; of them 12 participants received a placebo, and 12 other — 1/4 of the anticipated dose. To evaluate vaccine safety, participants were followed for adverse effects (according to WHO's classification [16]) for 20 weeks after the injection. Medical checkups included physical examination, ECG, blood and urine tests, chest X-ray, etc. In the second stage of the CS, we evaluated immunogenicity of GamTBvac in 12 volunteers who received two injections of the vaccine (1/4 of the anticipated dose each) separated by a 2-month interval. Dynamics of humoral and cell-mediated immune responses was monitored for 20 weeks following vaccination. The third stage of the CS aiming to determine the optimal dosage is still ongoing, and its results are not included in this work. It is being carried out in 24 other volunteers, of whom 12 have already received half of the anticipated dose and 12 other — the maximal (full) dose of the vaccine.

Expression and cloning of recombinant M. tuberculosis antigens

To induce expression of Ag85A and ESAT6-CFP10 antigens fused with dextran-binding domain [8], we used BL21(DE3) pLysS cells of *Escherichia coli*. The agitated cultures were grown in Lysogeny Broth (LB) containing 100 μg/ml ampicillin and 25 μg/ml chloramphenicol, at 37 °C. Once the cultures reached the optical density of 0.7–1 (at 600 nm), antigen expression was induced by adding to the suspension isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.5 mM and incubating the cultures at 30 °C for 4 hours, with agitation. Then the *E. coli* culture was centrifuged for 20 min at 6,000 g and 4 °C. The bacterial pellet was lysed in a buffer

Concentrations of antigens used for coupling to microspheres

Antigen	Selected concentration for coupling, μg per 10^6 microspheres	Microsphere region
DBD-ESAT6-CFP10	5	61
CFP-10	20	33
ESAT-6	10	25
DBD	20	55
DBD-Ag85A	20	67
Ag85A	20	42

containing 20 mM Tris-HCl pH 8, 200 mM NaCl, Triton-X100 0.1 %; after the cells were lysed, lysozyme was added to the buffer at a final concentration of 25 μg/ml; the mixture was incubated for 30 min at RT and then sonicated. Recombinant antigens were isolated either from the pellet after the lysis step or from supernatant. The lysate was centrifuged at 17,000 g for 20 min.

Ag85A-his8, DBD-his8, and ESAT6-his8 antigens were isolated from lysed bacterial cultures carrying the expression vector pET42b coding for *M. tuberculosis* proteins. The expressed proteins aggregated into insoluble inclusion bodies. Before dissolving the bodies in the buffer containing 8 M urea, we purified them three times in a lysing buffer to remove soluble and insoluble admixtures and pelleted by centrifugation. The micobacterial antigens dissolved in 8M urea were pulse-renaturated and run through the affinity column HisPrep FF 16/10 (GE, USA) for purification according to the manufacturer's protocol.

The antigen CFP10 was isolated from the lysates of bacterial cultures carrying the expression vector pTXB1 coding for CFP10. The soluble CFP10 protein was purified using the affinity matrix Chitin Resin (NEB, USA) according to the manufacturer's protocol.

Evaluation of cell-mediated immunity

To quantify the T-cells sensitive to M. tuberculosis antigens, the interferon-gamma release assay (IGRA) was performed [17, 18] modified as described in [19]. Briefly, 100 μl of whole blood were collected into a vacuum tube containing lithium heparin anticoagulant and the leukocyte fraction and introduced to 600 μl of the complete growth medium (90 % of medium 199, 10 % of fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES, 50 μg/ml gentamicin sulfate (all ingredients by PanEco, Russia). The resulting sample was divided into 4 aliquots. The recombinant antigens DBD-Ag85A and DBD-ESAT6-CFP10 contained in GamTBvac were added to 2 of the 4 aliquots at a final concentration of 50 μg/ml. One of the remaining aliquots was supplemented with concanavalin A (extracted from *Canavalia ensiformis*; Sigma-Aldrich, Germany) which non-specifically induces production of IFN by lymphocytes. This sample was used as a positive stimulation control. In the last aliquot 20 mM sterile salt-free TRIS, pH 7.5 was placed, and used as a negative control. In total, 4 stimulation reactions were run in separate tubes per blood sample: with antigens DBD-Ag85A and DBD-ESAT6-CFP10, and positive/negative controls. Blood-containing growth media with live lymphocytes, stimulating antigens and controls were incubated for 72 hours under sterile humid conditions at +37 °C. Upon incubation, interferon gamma was quantified using A-8752 gamma-interferon-ELISA-BEST kit (Vector-Best, Russia). Increased levels of interferon gamma as compared to the levels of its spontaneous production in the negative control were regarded as a positive response to stimulation [15, 19]. For each of 12 volunteers who participated in this stage of our study, blood was collected before vaccination (day 0) and on days 1, 42, 63, 98 and 140 after it.

Suspension immunoassay for measuring humoral immunity

For serology-based quantification of antibodies to Ag85A, ESAT6, CFP10 and DBD and their fusions DBD-Ag85A and DBD-ESAT6-CFP10, we used the obtained recombinant proteins and 6 xMAP-based monoplex assays (Luminex Corporation, USA) [20].

The optimal quantities of (fusion) antigens (5 to 20 μg per10^6 microspheres) were coupled to 6 microsphere sets (see the Table) through carbodiimide reactions according to the protocol described in The xMAP *Cookbook*, *3rd ed*. by Luminex [20].

Microspheres (1×10^6) were activated in 80 μl of the activation buffer (0.1 M NaH_2PO_4, pH 6.2) containing 10 μl of 50 μg/ml (in dH_2O) 1-ethyl-3-[3-dimethylaminopropyl carbodiimide hydrochloride (EDC) and 10 μl of 50 μg/ml (in dH2O) N-hydroxysulfosuccinimide sodium salt (s-NHS) for 20 min at 25 °C by rotation at 20 rpm. The activated microspheres were washed twice and resuspended in 500 μl of the coupling buffer (50 mM MES, pH 5.0), and the target antigens were added to the suspension. The microspheres were further incubated for 2 h in the dark at 25 °C, with mixing by rotation, and washed three times in the blocking buffer, then resuspended in 1 ml of the storage buffer and stored for at least 16 h until further analysis. The microspheres were counted using the automated cell counter TC20 (Bio-Rad Laboratories, USA). The blocking/storage buffer was PBS-TBN (PBS, 0.1 % BSA, 0.02 % Tween-20, 0.05 % NaN_3).

The indirect serological assay was run as recommended in [20]. Fifteen μl of PBS-TBN with 2,500 microspheres per region and 50 μl serum prediluted with PBS-TBN 50-fold (to a final concentration of 1 : 100) were placed into a well of a 96-well Microlon flat bottom clear polystyrene plate (Greiner, Austria). The mixture was incubated in the thremoshaker PST-60HL-4 (Biosan, Latvia) for 60 min at +25 °C and 800 rpm and then washed using the handheld magnetic separator MILLIPLEX (Merck Millipore, Germany). Briefly, 100 μl PBS-TBN were added into each well and the plate was left in the shaker for 30 seconds at 800 rpm for separation; 2 washing cycles were run (washing steps were the same throughout this part of the experiment). Then microspheres were resuspended in 50 μl PBS-TBN and combined with 50 μl of 5 μg/ml (in PBS-TBN) anti-human IgG goat antibodies conjugated with phycoerythrin (One Lambda/Thermo Fisher Scientific, USA). The final dilution of the conjugate in each well was 2.5 μg/ml. The suspension was incubated in the thermoshaker for 30 min at +25 °C and 800 rpm and washed. The washed microspheres were resuspended in 100 μl PBS-TBN. Results were processed using MAGPIX (Luminex, USA). For the analysis, we used a minimum of 100 microspheres of the same region per well.

Results of antibody quantification were expressed as MFI (median fluorescence intensity). For each sample, negative control (normal rabbit serum) was subtracted from the raw value. For each of 12 volunteers who participated in this stage of our study, blood was collected and antibodies to all studied antigens were quantified before vaccination (day 0) and on days 1, 42, 63, 98 and 140 after it.

Statistical processing

Data were statistically processed in MS Excel and GraphPad Prism 6. Significance of differences for humoral and cell-mediated responses was determined using the Wilcoxon matched-pairs test. Difference was considered significant at $p < 0.05$.

RESULTS

Safety of GamTBvac administered in the minimal dose

No (serious) adverse effects were registered in the course of our clinical study (Fig. 1). All abnormalities in lab tests and

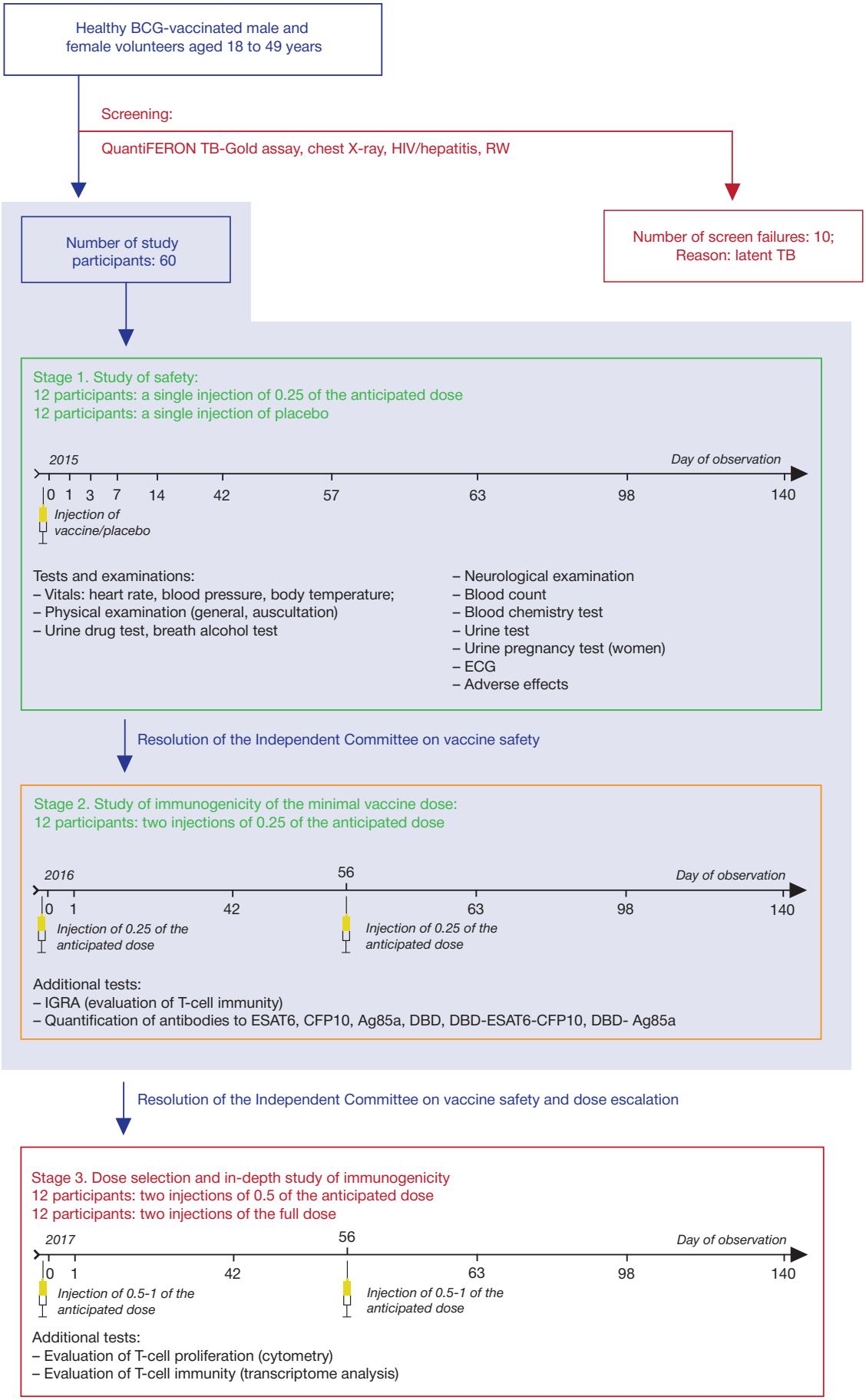

Fig. 1. The schematic of the study design. Stages 1 and 2 of the study reported in this article are shown in bold

ECG were found clinically insignificant by the experts of the authorized clinical facility [9]. Six participants reported post-vaccination redness at the injection site 24 h after the injection, persisting for no longer than 3 days. This adverse effect is described in the vaccine information leaflet and classified as mild by WHO [16]; therefore, it was regarded as clinically insignificant. In the light of the above, we conclude that ¼ of the anticipated GamTBvac dose [8] containing 0.006 mg of the antigen protein DBD-Ag85A (the mycobacterial protein Ag85A fused with dextran-binding domain), 0.006 mg of the antigen protein DBD-ESAT6-CFP10 (the mycobacterial protein ESAT6 fused with the mycobacterial CFP10, fused with dextran-binding domain), 2.5 mg of 500 kDa dextran; 0.125 mg of 500 kDa DEAE-dextran and 0.0375 mg of CpG-oligonucleotides (5'-ggGGGACGA:TCGTCgggggg-3') has a high degree of safety.

Cell-mediated immunity

Cell-mediated immunity plays a key role in protecting the host from tuberculosis, therefore, evaluation of vaccine immunogenicity should be in the first place account for the cell-mediated immune response to a vaccine [6, 21, 22]. Normally, immunogenicity is evaluated by measuring secretion of INF-γ in response to its stimulation with antigens contained in the vaccine. The most common methods used for this purpose are IGRA, ELISPOT (Enzyme-Linked ImmunoSpot) and flow cytometry. In this study we used a modified IGRA.

While studying INFγ secretion by T lymphocytes in response to the stimulation with recombinant *M. tuberculosis* antigens, we discovered that both antigens contained in the vaccine (DBD-Ag85A and DBD-ESAT6-CFP10) induce significantly increased production of INFγ (Fig. 2, A, B). A reliably increased response to DBD-Ag85A was observed after the first immunization, starting form day 42. Because Ag85A is expressed by *M. bovis* and all volunteers were previously vaccinated with BCG as recommended by the national immunization schedule, early cell-mediated response to Ag85A can be explained by a booster effect of GamTBvac [9]. The cell-mediated response to DBD-ESAT6-CFP10 became more pronounced a month after the second dose of GamTBvac, starting from day 98. On day 140 following the first vaccination, 10 (83 %) of 12 GamTBvac-vaccinated participants demonstrated a sustained cell-mediated response to all antigens contained in the vaccine, in comparison with that measured before the experiment.

Therefore, we conclude that the antigen composition of GamTBvac is immunogenic and induces T-cell response. Both antigen fusions have pronounced immunogenicity, although the temporal patterns of the immune response are different.

Humoral immunity

The temporal pattern of accumulation of class G antibodies to the antigens contained in GamTBvac (DBD-Ag85A and DBD-ESAT6-CFP10) reveals that both antigen fusions (to a greater or lesser extent) induce secretion of antibodies (Fig. 2, C–H), but the fusion DBD-ESAT6-CFP10 triggers a stronger humoral response (Fig. 2, C). Starting from day 63 after the first dose was administered, the antibody levels increased significantly. On the final day of the experiment (day 140), 11 (92 %) of 12 volunteers still had antibodies to the DBD-ESAT6-CFP10 fusion. Analysis of immunogenicity of its components revealed that both sense antigens CFP10 (Fig. 2, D) and ESAT6 (Fig. 2, E) stimulated production of IgG antibodies between days 98 and 140 of the experiment, respectively ($p^{**} = 0.0022$ and $p^{*} = 0.034$). Interestingly, DBD was also immunogenic, inducing an earlier response than the sense antigens, starting from day 63 (Fig. 2, F) after immunization.

Another fusion antigen DBD-Ag85A demonstrated a relatively low immunogenicity (Fig. 2, G). For this antigen, significant differences were observed staring from day 98 ($p^{*} = 0.041$) following the immunization. However, no significant associations ($p > 0.05$) between vaccination and production of IgG antibodies to Ag85A were observed (Fig. 2, H).

DISCUSSION

Phase I of the clinical study of GamTBvac has demonstrated that the vaccine has an acceptable safety profile when administered in the studied doses twice to healthy volunteers without latent tuberculosis previously vaccinated with BCG. No serious adverse effects were reported. Redness was observed in 6 participants at the injection site 24 hours after the injection, persisting for no longer than 3 days. This adverse effect is described in the vaccine information leaflet and is classified as mild by WHO [16]; therefore, it was regarded as clinically insignificant.

Our study demonstrates that humoral response was induced in 10 (83 %) of 12 volunteers who received the GamTBvac vaccine. The fusion DBD-Ag85A enhanced the booster effect already observed after the first injection (Fig. 2, A). This effect is apparently associated with Ag85A, because the second antigen DBD-ESAT6-CFP10 elicited the immune response only after the second injection (Fig. 2, B). In our study we did not evaluate separate contributions of each fusion component to the cell-mediated immune response. However, these contributions were studied in depth in our previous experiments conducted in mice [8]. We showed that Ag85A and ESAT6 were the most immunogenic, while CFP10 induced a less pronounced response, although still significantly contributing to the booster effect of the vaccine. We also demonstrated that DBD did not make a significant contribution to the activation of the cell-mediated immunity [8]. This is consistent with the results obtained by other researchers. For example, a few clinical trials of recombinant vaccines H1, H4 and H56 conducted by the State Serum Institute (Copenhagen, Denmark) showed that Ag85 and ESAT6 are highly immunogenic [21–23], triggering a sustained cell-mediated response that was observed half a year after the first immunization. Our study demonstrates that GamTBvac also induces formation of cell memory observed through day 140 of the study.

Cell-mediated immunity has been conventionally thought to have a primary role in protection against tuberculosis, but recently evidence has started to emerge of a more significant role of humoral immunity [6, 24]. In this light, studies of the activation of humoral immunity by TB vaccines are becoming increasingly important. GamTBvac induced humoral response to all components of the antigen fusion DBD-ESAT6-CFP10 (Fig. 2, C–F). The earliest and strongest response was observed for antigen CFP10 (Fig. 2, D), indicating that CFP10 induces both cell-mediated and humoral immunity. A statistically reliable response to ESAT6 occurs later and is less intense, but it appears to make its own contribution to the total immunogenicity of DBD-ESAT6-CFP10 (Fig. 2, C, E).

Obviously, a sustained strong humoral response to the "auxiliary" antigen DBD (Fig. 2, F) cannot associated with protection against TB, but anti-DBD immunoglobulins are potential candidate serologic markers that can be employed by population studies or precision medicine, if patient's immunization status be unknown.

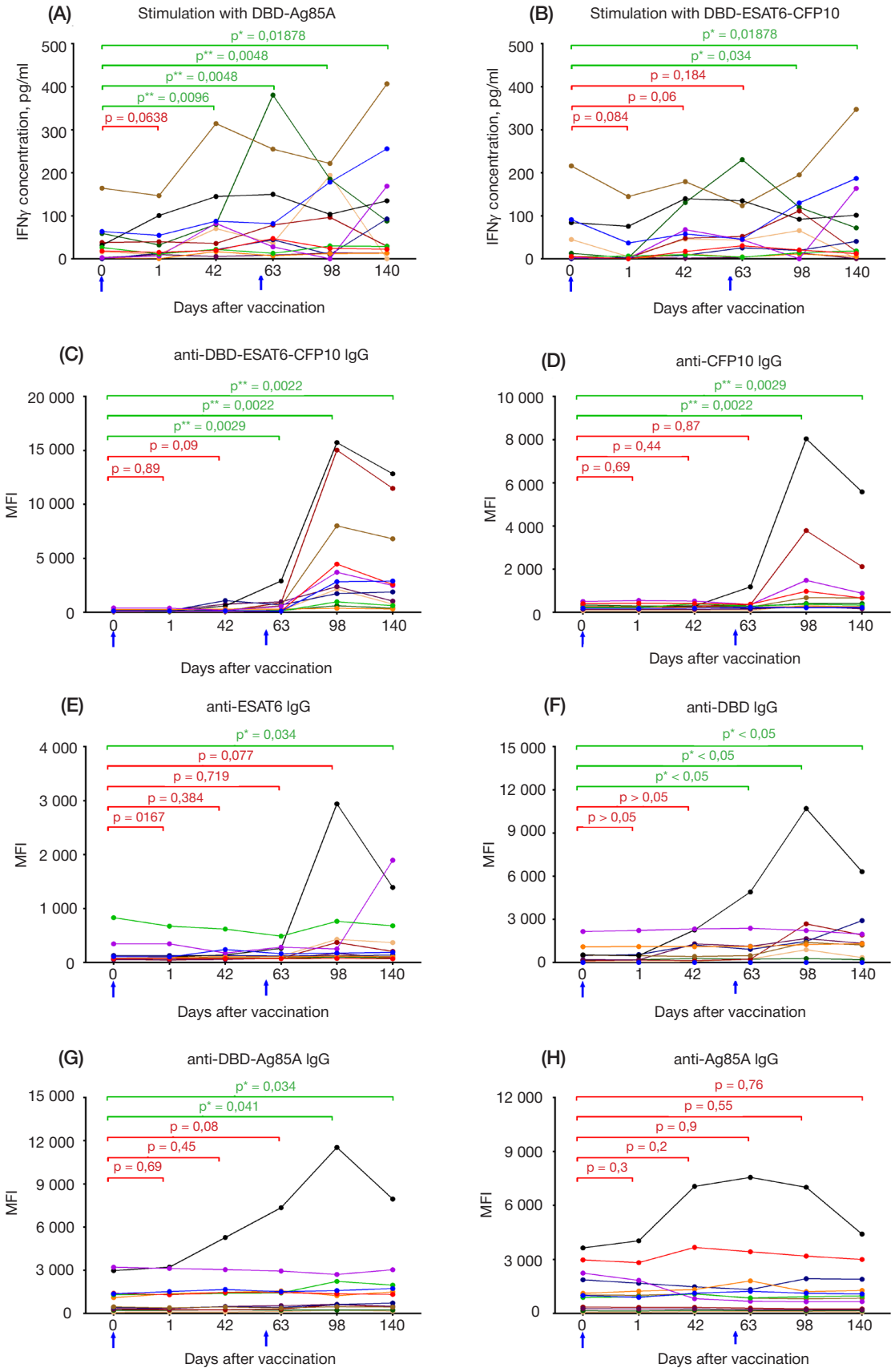

Fig. 2. Immunogenicity of the GamTBvac vaccine in 12 volunteers.
(A), **(B)** Evaluation of cell-mediated immunity. For the analysis we employed a modification of IGRA [19] that measures INFγ secretion by whole blood lymphocytes in response to stimulation with antigens DBD-Ag85A **(A)** and DBD-ESAT6-CFP10 **(B)**. The value of spontaneous IFNγ secretion was subtracted from the raw value.
(C)–**(H)** x-MAP-based quantification of antibodies to the antigen fusion **(C)** and its components: CFP10 **(D)**, ESAT6 **(E)**, DBD **(F)**, and DBD-Ag85A **(G)** and its main component Ag85A **(H)**. Statistical significance was calculated using the Wilcoxon matched-pairs test. Significant differences over the observation period (*p < 0.05 and **p < 0.01) are shown in green, in comparison with day 0. Red p-values indicate the absence of significant differences. Vaccination days are represented by blue arrows

Surprisingly, Ag85A had a low humoral immunogenicity (Fig. 2, 3). This antigen seems to make a zero contribution to the total immunogenicity of the fusion DBD-Ag85A (Fig. 2, G). Previously, Ag85A was shown to induce high antibody titers both in BCG-unvaccinated and vaccinated animals [8]. The absence of humoral response may be explained by the incorrect folding of Ag85A used for coupling with xMAP microspheres. On the other hand, some of the volunteers had stably high antibody titers throughout the study, which may disprove this supposition. Possibly, increased antibody titers induced by BCG vaccination rapidly eliminate small amounts of antigen proteins without triggering the booster effect. Each of these hypotheses needs to be tested further.

As the role of humoral immunity in patients with tuberculosis is becoming increasingly acknowledged, preclinical and clinical trials of TB vaccines should focus more on the contribution of each vaccine component to the elicited humoral response [24]. Unfortunately, the majority of studies of TB vaccines do not report any findings about antibody response [22, 23] or do not differentiate between contributions made by individual antigens [21]. Our results demonstrate that effects of antigens contained in GamTBvac can be directly opposite in terms of establishing the cell-mediated and humoral immunological memory.

CONCLUSIONS

The studied recombinant vaccine GamTBvac has a sufficient degree of safety and activates humoral and cell components of the immune system leading to the establishment of immunological memory. Our study demonstrates that 1/4 of the anticipated vaccine dose induces sustained cellular and humoral responses. We anticipate that stage 3 of our study, which is still ongoing, will be successful and that we will be able to demonstrate the strong immunogenicity of the vaccine using the most advanced techniques, including flow cytometry and transcriptome analysis. We also hope to carry out Phase IIb to confirm vaccine effectiveness.

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ADAPTIVE IMMUNITY AND GENETIC ASPECTS OF TUBERCULOSIS IN CHILDREN

Plekhanova MA ✉

Department of Pediatrics, Center of Continuing Professional Education and Retraining,
Omsk State Medical University, Omsk, Russia

Cell-mediated immunity and the cytokine interferon gamma (IFNγ) have an important role in promoting host resistance against tuberculosis-causing mycobacteria (TBM), but the exact mechanism of developing immunity against tuberculosis (TB) is unknown. In this work we evaluate the immune response in TB and the association between *IFNG* gene polymorphism *rs2069705* (T-1488C) and the intensity of specific immune reactions in children. The study was conducted in 310 children below 18 years distributed into 3 groups: the TB group included 110 children with TB confirmed by medical evaluation; the LTB group consisted of 156 children with latent infection; and the NTB group was represented by 44 non-infected children. A few immunoassays and molecular-genetic tests were performed; specifically, we evaluated the immune status of patients and the distribution of genotypic frequencies of the studied polymorphism, in the context of previous vaccination against TB. The cell-mediated immune response was mild in children with LTB, while in children with TB inflammation showed signs of chronicity due to the lack of functional activity of immune cells ($p < 0.05$). We also measured IFN-γ synthesis induced by specific mitogens (PPD-L, CFP32B, Rv2660c, ESAT6, 85a and ESAT6-CFP10), only to detect attenuation of the immune response in patients with TB, which was associated with the heterozygous *rs2069705* variant ($p < 0.05$). Children with homozygous TT and CC genotypes demonstrated a more pronounced immune response. Low effectiveness of the TB vaccine was shown to be associated with the heterozygous genotype (50 %), while its high effectiveness was associated with the homozygous T genotype (40 %), possibly indicating the protective role of the latter. Our findings suggest that the studied polymorphism (specifically, its heterozygous variant) can be a predictive marker of TB in children.

Keywords: Mycobacterium tuberculosis, tuberculosis, immune response, interferon gamma, antigen, PPD-L, CFP32B, Rv2660c, ESAT6, 85a, ESAT6-CFP10, *IFNG*, polymorphism, children

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✉ **Correspondence should be addressed:** Maria Plekhanova
ul. Lenina, d. 12, Omsk, Russia, 644099; dina-plus@mail.ru

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АДАПТИВНЫЙ ИММУНИТЕТ И ГЕНЕТИЧЕСКИЕ АСПЕКТЫ ПРОГРЕССИРОВАНИЯ ТУБЕРКУЛЕЗНОЙ ИНФЕКЦИИ У ДЕТЕЙ

М. А. Плеханова ✉

Кафедра педиатрии ДПО, Центр повышения квалификации и профессиональной переподготовки специалистов,
Омский государственный медицинский университет, Омск

Клеточный иммунитет и цитокин интерферон гамма (ИФН-γ) имеют важное значение для формирования устойчивости организма к микобактериям туберкулеза (МБТ), но точный механизм появления иммунитета к туберкулезу (ТБ) неизвестен. В исследовании оценивали иммуный ответ при развитии ТБ и связь полиморфизма *rs2069705* (T-1488C) гена *IFNG* с выраженностью специфических иммунологических реакций у детей. Участниками исследования стали 310 детей до 18 лет, распределенные по 3 группам: группа ТБ — 110 детей с установленным по результатам комплексного обследования ТБ; группа ЛТИ — 156 детей с установленной латентной туберкулезной инфекцией; группа НТ — 44 ребенка, не инфицированные МБТ. Проводили иммунологические и молекулярно-генетические исследования, в частности, оценивали иммунный статус пациентов и распределение частот генотипов по изучаемому полиморфизму, в том числе в контексте эффективности вакцинирования против ТБ. Иммуный статус детей с ЛТИ характеризовался лишь слабо выраженной активацией клеточного иммунитета, а детей с ТБ — появлением у воспалительного процесса черт хронического за счет недостаточной функциональной активности клеток иммунной системы ($p < 0,05$). При оценке иммуного ответа по уровню синтеза ИФН-γ, индуцированного специфическими митогенами (PPD-L, CFP32B, Rv2660c, ESAT6, 85a и ESAT6-CFP10), установили снижение ответа у больных ТБ, которое было ассоциировано с гетерозиготным генотипом по полиморфизму *rs2069705* гена *IFNG* ($p < 0,05$). При гомозиготных генотипах TT и CC ответ усиливался. Также установили, что низкая эффективность противотуберкулезной вакцинации также связана с гетерозиготным генотипом (50 %), а высокая — с генотипом, гомозиготным по аллелю T (40 %), что может свидетельствовать о его протективной роли. Полученные результаты указыают на то, что изучаемый полиморфизм (гетерозиготный генотип) можно рассматривать в качестве маркера развития туберкулезной инфекции у детей.

Ключевые слова: микобактерии туберкулеза, туберкулез, иммунный ответ, интерферон гамма, антиген, PPD-L, CFP32B, Rv2660c, ESAT6, 85a, ESAT6-CFP10, *IFNG*, полиморфизм, дети

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✉ **Для корреспонденции:** Плеханова Мария Александровна
ул. Ленина, д. 12, г. Омск, 644099; dina-plus@mail.ru

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The research efforts of the recent years have proved the important role cellular immunity and interferon gamma (IFNγ) play in protecting human body from mycobacterium tuberculosis (MBT), but the mechanisms of development of immunological competence against tuberculosis (TB) remain poorly understood [1]. It is known that proteins secreted by MBT at early stages of infection support proliferation of lymphocytes producing IFNγ [2]. A number of studies states that TB patients with nonresistant *IFNG* genotype have the level of synthesis of this cytokine correlated to the severity of the disease [3, 4].

The search for candidate genes causing congenital and adaptive immunity disorders in the presence of TB is also underway [1, 5–9]. Previously, *IFNG* gene polymorphism in development of various diseases, including tuberculosis, received much attention [3, 10–13].

Studying immunological and genetic factors affecting antituberculous immunity ensures better understanding of the underlying pathogenesis of the disease and, what is more, allows optimization of the prevention approaches. This research aimed to investigate the immune response to TB development and the relationship between polymorphic variant *rs2069705* (T-1488C) of *IFNG* gene and the expression of specific immunological reactions in children.

METHODS

This prospective study was conducted in 2014–2016. 310 patients, all under 18 years of age, took part in it. They were divided into 3 groups depending on the TB status: TB group — 110 children, confirmed TB, mean age of 9.5 ± 0.5, every second child (46.6 %) under 8 years of age; LTB group — 156 children, latent TB confirmed (tuberculin diagnostics), mean age of 6.6 ± 0.3, 70.5 % of children under 8 years of age; NTB group — 44 children, no TB infection, mean age of 3.1 ± 0.4, 95.5 % of children under 8 years of age. Genderwise, the groups were not different ($p > 0.05$).

All children underwent specific immunoassays; 169 of them were also subjected to molecular-genetic tests. To make the comparison valid, only the immune status data describing children under 8 were compared.

TB diagnosing included various laboratory, bacteriological, molecular-genetic tests, radiological examinations. The results of tuberculin diagnostics were also taken into account: Mantoux tests with 2 tuberculin units (TU) of tuberculin (PPD-L) and recombinant tuberculosis allergen tests (RTA) Diaskintest (Generium, Russia), which contain 0.2 μg of CFP10-ESAT6 recombinant protein. A central medical-control commission checked and confirmed diagnoses.

In the TB group, 23 children were diagnosed with infiltrative pulmonary tuberculosis, 38 — with primary tuberculosis, 46 — with intrathoracic lymph nodes tuberculosis (one patient had it combined with the left proximal bronchus lesion and there were cases of focal pulmonary TB, disseminated pulmonary TB and pleuresia tuberculosa, one of each). In 4 cases, TB also affected other organs: peripheral lymph nodes, humerus, intestine. Also in 4 cases, TB caused complications (atelectasis, exudative pleurisy, pulmonary dissemination, hemoptysis). 60.9 % (n = 67) of patients had TB at the infiltration stage, 10.9 % (n = 12) showed disintegration and seeding phase, 0.9 % (n = 1) — sealing and resorption, 26.4 % (n = 29) — calcination phase. Bacterioexcretion was registered in 12 cases (10.9 %); 6 of them proved resistant to the basic antituberculosis drugs.

In the LTB group, 75 children were diagnosed with early primary tuberculosis (EPT), and 81 children had MTB for more than one year.

In the NTB group, 21 child had post-vaccination allergy and 23 showed positive tuberculin anergy.

TB group participants had the infiltrate measure the average of 12.6 ± 0.4 mm (95 % CI 11.8–13.5 mm) for the Mantoux test and 14.95 ± 0.5 mm (95 % CI 13.9–16.0 mm) for the RTA test. The mean values for the same tests in the LTB group were 10.3 ± 0.3 mm (95 % CI 9.7–10.9 mm) and 1.9 ± 0.4 mm (95 % CI 1.2–2.65 mm) respectively. As for the NTB group children that had postvaccinal allergy, the Mantoux test there brought the average results of 4.1 ± 0.4 mm (95 % CI 3.2–5.0 mm); 9 (42.9 %) cases were confirmed positive reactions, the rest were questionable. All children in this group returned negative reaction to the RTA test.

We had the data on TB vaccination of children. BCG administration site scar and Mantoux test a year after vaccination helped determine its effectiveness: when the scar was 4–10 mm long and tuberculin response positive, the vaccination was regarded a success, in the absence of the scar and negative reaction to tuberculin it was considered ineffective, all other cases fell into minimally effective category. In the TB group, 108 children (98.2 %) were vaccinated, but only for 31 of them the vaccination was effective. In the LTB group, 155 children (99.4 %) received vaccination and for the most of them (n = 97) it was effective. 36 children (81.8 %) of the NTB group were vaccinated; it was effective in every fourth case.

Patients went through immunoassays and molecular-genetic tests when admitted to the special in-patient department of the TB dispensary. The tests were made at the Omsk R&D Institute for Natural Focal Infections (Russia).

Immune status was assessed with the standard level I immunological screening tests: measurement of immunoglobulin levels in the blood serum (IgG, IgA, IgM and IgE) through enzyme-linked immunoassay; evaluation of neutrophil function through measurement of their ability to absorb inert latex particles and two variations of nitroblue tetrazolium (NBT) test, spontaneous and stimulated; evaluation of subpopulation composition of T-cells (CD) using a panel of monoclonal antibodies (DAKO, Denmark). We have also measured the content of spontaneously synthesized interferon gamma and IFNγ the synthesis of which was induced by specific antigens (PPD-L, CFP32B, Rv2660c, ESAT6, 85a, ESAT6-CFP10) for 72 h. Enzyme immunoassay system by Vector-Best (Russia) was used for the purpose. Antigens were isolated by the translational biomedicine lab of the Department of genetics and molecular biology of bacteria of N. F. Gamaleya Scientific Research Institute of Epidemiology and Microbiology (Moscow, Russia) [14].

DNA-blood reagent kit (TestGen, Russia) helped isolate DNA from blood serum; iQ5 amplifier (BioRad, USA) and a set of polymerase chain reaction reagents (FLASH format, by TestGen) were used to identify polymorphic marker *rs2069705* of *IFNG* gene, all following instructions provided by the manufacturers. Allelic Discrimination software supplied by the manufacturer of the amplifier enabled analysis of genotypes. The frequency distribution of genotypes at the examined loci was checked for compliance with the Hardy–Weinberg law.

OpenEpi v3.0 software calculated the minimal sample size ensuring conclusiveness of the empirical data obtained. The reliability of differences between groups was determined through nonparametric Kruskal–Wallis (H), Mann–Whitney (U) and χ^2 tests. The differences were considered significant at $p < 0.05$. We have also calculated the odds ratio (OR): if the chance (risk) was above 1, development of the disease was considered statistically significant. OpenEpi v3.0 and Statistica v6.0 software were used to process the data obtained.

The study got the approval of Omsk State Medical Academy ethics committee (Minutes no. 51 of October 10, 2012). Parents or legal representatives have signed voluntarily informed consent forms and thus approved participation of their children in the study.

RESULTS

Table 1 shows the comparative analysis of results of clinical and laboratory examinations/tests of children from TB and LTB groups. Children from the LTB group suffered from intoxication syndrome less often (9 %) than the TB group patients (19.1 %) ($p = 0.008$), but they had more of nasal breathing disorders caused by adenoid vegetations (16 % vs. 1.8 %, $p = 0.00008$), chronic infection foci (18.6 % vs. 10 %, $p = 0.027$), respiratory allergies manifestations (15.4 % vs. 0 %, $p = 0.000008$) and excess weight (10.3 % vs. 4.5 %, $p = 0.044$). Such clinical symptoms as hepato- and splenomegaly (OR 2.583 and 3.800, respectively) and body mass deficiency (OR 1.898) were significant in the TB group. Lab tests have also shown significance of anemia (OR 1.872), accelerated ESR (OR 2.255), lymphocytosis (OR 1.634) and eosinophilia (OR 5.371). It should be noted that only 4 cases of eosinophilia (out of 40) resulted from parasitic invasions.

Table 2 shows the results of assessment of the immune status of all children that participated in the study. There were no significant differences in immunological parameters describing the status of children constituting LTB and NTB groups ($p > 0.05$). The exception here was the content of spontaneously synthesized IFNγ ($p < 0.05$). In all groups, the numbers of leukocytes have increased slightly (compared to the reference values). Leukocytes reflect the state of cellular immunity, so the increase can signal of its activation due to nonspecific processes. At the same time, the differences were significant for CD16, HLA DR, and spontaneously synthesized IFNγ. In the TB group, activation of humoral immunity was observed: within the reference values, the volumes of IgG, IgA have grown, and IgE have shown a significant increase to 306.6 ± 130.7 IU/ml. Phagocytic activity of cells has also increased significantly, while neutrophils' reserve capacity has decreased. The changes of all the aforementioned values were statistically significant compared to those seen in the NTB group.

Thus, the immune status of children from the LTB group was the same to that of children not hosting MTB; at that, cellular immunity has activated slightly. In the TB group, no immunodeficiency development signs were observed, but the changes that did manifest themselves were those peculiar to an inflammatory process associated with TB. The process had features of a chronic one, primarily due to insufficient functional activity of cells and insufficient production of interferon gamma.

To better understand the contribution IFNG gene makes to the immunological defense against MBT, we investigated its polymorphism *rs2069705*. 51.9 % of children from the TB group had a heterozygous genotype, so it was associated with the disease (OR 1.885, 95 % CI 1.019–3.487). Be it secondary (65 %) or primary (47.5 %) TB, the genotype was there, which suggests a higher risk of the disease development regardless of its genesis. Heterozygous genotype was also associated with the development of infiltrates (OR 1.737), signs of lung tissue destruction (OR 1.458), dissemination (OR 1.75) and pleural effusion (OR 1.9), bacterial excretion (OR 1.458) and such clinical manifestations of tuberculosis as paraspecific reactions (OR 2.059), peripheral lymphadenopathy (OR 2.4), bodyweight deficit (OR 1.429), hepato- and splenomegaly (OR 5.5), anemia (OR 2.059), accelerated ESR (OR 3.4).

Given the association of *IFNG* gene polymorphism *rs2069705* with adaptive immunity to tuberculosis, we evaluated the genotype-wise effectiveness of BCG vaccination in children affected by primary TB at its early stage (n = 32). In 12 cases, the vaccination was minimally effective or ineffective; half of those patients had heterozygous genotype. The probability of minimal effectiveness of vaccination was 59.38 % (95 % CI 42.23–74.62 %). Homozygous genotype (T allele) was more common (40 %) among children for whom the vaccination was effective (n = 20).

Table 1. Results of clinical and laboratory examinations/tests of children from TB and LTB groups

Symptoms	TB group (n = 110), abs. (%)	LTB group (n = 156), abs. (%)	χ^2 criterion; p-value	OR
Intoxication syndrome	21 (19.1)	14 (9)	5,778; 0.008	2,393
Bronchopulmonary syndrome	10 (9.1)	12 (7.7)	0.166; 0.342	1,2
Paraspecific reactions	21 (19.1)	36 (23.1)	0.609; 0.218	0,787
Peripheral lymphadenopathy	18 (16.4)	19 (12.2)	0.943; 0.166	1,411
Hypertrophy of palatine tonsils, I-II degree	26 (23.6)	42 (26.9)	0.366; 0.273	0,84
Adenoids	2 (1.8)	25 (16)	14.28; 0,00008	0,097
Caries	6 (5.5)	14 (9)	1.149; 0.143	0,585
Hepatomegaly	7 (6.4)	4 (2.6)	2.349; 0.063	2,583
Splenomegaly	10 (9.1)	4 (2.6)	5,512; 0.009	3,8
Chronic infection foci	11 (10)	29 (18.6)	3,726; 0.027	0,487
Respiratory allergies	0	24 (15.4)	18.6; 0.000008	0
Body weight deficiency	24 (21.8)	20 (12.8)	3.783; 0.026	1,898
Excess body weight	5 (4.5)	16 (10.3)	2.894; 0.044	0,417
Anemia	16 (14.5)	13 (8.3)	2.563; 0.054	1,872
ESR acceleration	25 (22.7)	18 (11.5)	5.959; 0.007	2,255
Leukocytosis	16 (14.5)	28 (17.9)	0.541; 0.231	0,778
Lymphocytosis	43 (39.1)	44 (28.2)	3.473; 0.031	1,634
Monocytosis	19 (17.3)	25 (16)	0.073; 0.394	1,094
Eosinophilia	40 (36.4)	15 (9.6)	28.14; <0.0000001	5,371

Table 2. Results of immunoassay of blood of children from TB, LTB and NTB groups

Parameter	Reference value	TB group		LTB group		NBT group	
		n	M ± SEM	n	M ± SEM	n	M ± SEM
CD3. %	54–82	15	65.8 ± 1.3	16	66.2 ± 1.3	10	69.1 ± 1.7
CD3, abs.	1.65	15	1.8 ± 0.1	9	2.2 ± 0.3	8	2.1 ± 0.2
CD4, %	30–50	15	40.4 ± 1.5	16	39.0 ± 1.3	10	40.8 ± 1.0
CD4, abs.	0.92	15	1.1 ± 0.1	9	1.2 ± 0.15	8	1.2 ± 0.1
CD8, %	18–38	15	29.4 ± 1.4	16	26.9 ± 1.2	10	28.9 ± 1.4
CD8, abs.	0.6	15	0.8 ± 0.06	9	0.9 ± 0.15	8	0.9 ± 0.09
CD16, %	6–18	14	11.4 ± 0.5**	20	13.4 ± 0.6	10	17.1 ± 2.7
CD16, abs.	0.31	14	0.3 ± 0.03**	15	0.4 ± 0.04	10	0.6 ± 0.1
CD20, %	6–22	15	16.2 ± 1.5	12	16.8 ± 1.75	3	14.0 ± 1.2
CD20, abs.	0.2	15	0.5 ± 0.07	5	0.6 ± 0.1	-	-
HLA DR, %	14–25	15	25.9 ± 1.4**	20	23.7 ± 1.6	9	21.8 ± 1.1
HLA DR, abs.	0.33	15	0.7 ± 0.07	15	0.7 ± 0.09	9	0.7 ± 0.07
IFNγ sp., Pg / ml	0.16–10	50	21.1 ± 5.7**	110	20.5 ± 3.0***	43	12.9 ± 1.7
IgG, g / l	8.12–16.14	19	11.7 ± 0.6*.**	110	9.7 ± 0.2	43	8.9 ± 0.3
IgA, g / l	0.75–3.17	19	1.6 ± 0.1*.**	110	1.2 ± 0.06	43	1.0 ± 0.08
IgM, g / l	0.69–3.00	19	1.4 ± 0.2	110	1.15 ± 0.05	43	1.2 ± 0.08
IgE, IU / ml	< 150	19	306.6 ± 130.7*	108	79.3 ± 12.6	23	81.9 ± 25.5
Phagocytosis with latex, %	52–95	15	68.5 ± 3.1**	107	65.7 ± 1.4	33	53.3 ± 2.7
HCT test:							
– spontaneous, %	6–12	16	14.4 ± 1.9**	109	20.9 ± 1.3	41	24.4 ± 1.6
– stimulated, %	–	16	37.8 ± 3.2**	109	46.5 ± 2.2	41	56.5 ± 3.3

Note. * — $p < 0.05$ when comparing results in TB and LTB groups (Mann–Whitney test, U); ** — $p < 0.05$ when comparing results in TB and NTB groups (Mann–Whitney test, U); *** — $p < 0.05$ when comparing results in LTB and NTB groups (the Mann–Whitney test, U).

Ozhegova et al. [7] have established that polymorphism T-1488C of *IFNG* gene defines its expression level. Therefore, we assumed there is a connection between the genotypes studied and the level of interferon gamma synthesis. Analysis of the content of spontaneously synthesized IFNγ did not reveal any significant differences dependent on genotype; this is true for both LTB ($H = 1.663$, $p = 0.435$) and TB ($H = 4.810$; $p = 0.090$) groups. Analysis of the content of IFNγ synthesized through induction by specific antigens [15] showed that in the development of TB, there is a relationship between genotype and level of cytokine synthesis: with heterozygous genotype, the content of IFNγ decreased significantly under the influence of CFP32B, Rv2660c, ESAT6, Ag85a antigens (Table 3). With this in mind, we have also analyzed the frequency of negative results — no response to induction in children with TB (Table 4) — and established the relationship with heterozygous genotype. Its association with decelerated IFNγ synthesis when induced with PPD-L, CFP32B, Rv2660c, ESAT6, 85a and ESAT6-CFP10 antigens was confirmed.

DISCUSSION

The vast majority of people carrying MTB show no symptoms of tuberculosis, and although they are not contagious, they run a risk of developing an active form of TB. According to experts, the risk of TB reactivation during the lifetime of an LTB patient is 5–10 %. Often, the switch from latent to active happens within 5 years from the day of infection [16, 17]. Nevertheless, a number of researchers believes the level of this risk depends on several factors, the most important of which is the body's immune status [18–21]. The results of our study suggest presence of a specific inflammatory process and absence of secondary immunodeficiency.

We studied IFNγ, the main function of which is immunoregulation, including macrophages activation, enhancement of Th-1 mediated response, induction of expression of MHC class II antigens on antigen-presenting cells, etc. [22]. With cellular immunity activation in the background, IFNγ producers are activated Th1-lymphocytes (the main activation marker is HLA DR) and natural killer cells (CD16). Therefore, reduced numbers of those natural killer cells in the presence of TB could lead to lowered levels of cytokine synthesis, and IFNγ deficiency could cause a decrease in activity of cytotoxic cells. This could explain the growing volume of spontaneously synthesized IFNγ seen even when TB is latent ($p < 0.05$); at the same time, we can assume that the antigen load was not sufficient to trigger hyperactivation of cellular response. During the period of development of TB, this indicator remained at the level typical of LTB ($p > 0.05$). Given the increased volume of spontaneously synthesized IFNγ, one could expect a lower content of IgE, as IFNγ, being the product of Th1-lymphocyte, inhibits proliferation of Th2-lymphocytes and IL4-induced Ig to IdE synthesis switch and supports IgG2 synthesis instead [23]. However, we have witnessed high levels of IgE synthesis during TB development, which may indicate inadequate IFNγ production and onset of chronic inflammation. A number of researchers have also established a direct relationship between the appearance of chronic bacterial or fungal infection foci and hyperproduction of IgE [24–26].

IFNγ is also considered to be the most important factor in macrophage activation [22]. Macrophages lyse MTB and provide antimycobacterial protection; in particular, they regulate synthesis of pro- and anti-inflammatory cytokines [27, 28]. However, the balance between cells and mediators required to destroy MTB and prevent lung pathology is still unclear; the issue is subject of other research papers in progress [29]. In

Table 3. Level of interferon gamma synthesis induced by specific antigens, depending on the genotype (polymorphic variant *rs2069705* (T-1488C) of *IFNG* gene) in TB group children

Antigen	*IFNG* gene polymorphic variant *rs2069705* (T-1488C)		Mann–Whitney test (U); p-value
	TS	TT and CC	
	Me (Q25%; Q75%), n = 42	Me (Q25%; Q75%), n = 39	
PPD-L	1001.6 (698; 1200)	1380.7 (741.5; 1294.5)	703.5; 0.275
CFP32B	69.8 (10.2; 68)	95.8 (21; 122.5)	554.5; 0.018
Rv2660c	102.6 (12.2; 83)	149.6 (37.5; 195.5)	513; 0.006
ESAT6	101.5 (10.9; 57.4)	112.8 (26; 173)	523.5; 0.005
85a	65 (0.2; 40)	90.3 (6.5; 101)	549.5; 0.016
ESAT6-CFP10	440.4 (139; 761)	549.5 (187; 1133)	768; 0.630

Table 4. Frequency of negative reactions to specific antigens depending on genotype (polymorphic variant *rs2069705* (T-1488C) of *IFNG* gene) in TB group children

Antigen	*IFNG* gene polymorphic variant *rs2069705* (T-1488C)				p-value	OR	95 % CI
	TS (n = 42)		TT and CC (n = 39)				
	abs.	%	abs.	%			
PPD-L	3	7.1	1	2.6	0.202	2.923	0.291–29.35
ESAT6-CFP10	8	19	3	7.7	0.068	2.824	0.691–11.53
ESAT6	40	95.2	24	61.5	0.0001	12.5	2.628–59.47
Rv2660c	37	88.1	30	76.9	0.092	2.22	0.672–7.33
CFP32B	34	81	19	48.7	0.0012	4.474	1.656–12.08
85a	34	81	19	48.7	0.0012	4.474	1.656–12.08

our study, we saw a decrease in phagocytic activity of cells (decrease in reserve capacity of neutrophils) against the backdrop of developing TB, which may also point to inadequate production of IFNγ.

Averbakh et al. proposed a hypothesis stating there are genes in lymphocytes that control activation of cells synthesizing mediators and that a depression of one part of the genome can mean a depression of its another part, which may lead to disruption of intercellular interaction in the presence of TB [30]. The researchers are trying to establish genetic risk factors affecting TB infection and development [8, 9]. In particular, they are actively studying the *IFNG* gene associated with production of IFNγ cytokine [3,9] and T-1488C polymorphism that affects the synthesis of regulatory protein [7]. In the context of our study, the marker of high TB risk was its heterozygous genotype at the studied genetic locus (OR 4.667, 95 % CI 1.236–17.62, p = 0.008), regardless of the disease genesis (primary or secondary). Low effectiveness of BCG seen in the group with primary tuberculosis infection at its early stage is an indirect proof thereof. Some researchers also believe the low effectiveness of vaccination is a TB risk factor [18, 31]. We have also determined that the studied *IFNG* gene polymorphism (heterozygous genotype) influences the immune response to individual mycobacterial antigens when TB is developing: we have seen a significantly weaker response to early stage TB proteins — CFP32B, Rv2660c, ESAT6, 85a [15], and a slightly decreased level of IFNγ synthesis with PPD-L and ESAT6-CFP10 induction, which gives a yet another reason to look at these antigens in the context of TB. We have also established that homozygous genotype (T allele) means better protection against TB and found it affects development of antituberculous immunity in children.

The results of this study allow taking the *IFNG* gene polymorphism (T-1488C) as an additional genetic risk factor contributing to the development of tuberculosis infection in children and one of the reasons behind inadequate functional activity of cells regulating synthesis of IFNγ.

CONCLUSIONS

We studied the immune response to development of TB and seen activation of cellular immunity and insufficient functional activity of cells when TB turns from latent to active. The main adaptive immunity cytokine considered was IFNγ.

We have established that *IFNG* gene polymorphism T-1488C affects the severity of specific immunological reactions. Heterozygous genotype implies inadequate production of cytokine at the early stage of TB development. Homozygous genotype (T allele) means better protective immunity against the disease.

We have established that heterozygous genotype of the *IFNG* gene's polymorphic version (*rs2069705*) bears relation to TB switching from latent to active in children, which allows taking this genotype as an additional risk factor.

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A PROTOCOL OF DEVELOPMENT OF A SCREENING ASSAY FOR EVALUATING IMMUNOLOGICAL MEMORY TO VACCINE-PREVENTABLE INFECTIONS: SIMULTANEOUS DETECTION OF ANTIBODIES TO MEASLES, MUMPS, RUBELLA AND HEPATITIS B

Mazunina EP[1] ✉, Kleymenov DA[1], Manuilov VA[1,2], Gushchin VA[1,3], Tkachuk AP[1]

[1] Laboratory of Translational Biomedicine,
N. F. Gamaleya Federal Research Centre for Epidemiology and Microbiology, Moscow, Russia

[2] Institute of Molecular Medicine,
I. M. Sechenov First Moscow State Medical University, Moscow, Russia

[3] Department of Virology, Faculty of Biology,
Lomonosov Moscow State University, Moscow, Russia

Multiplex screening assays for measuring antibodies to vaccine-preventable infections are routinely used in large-scale seroepidemiological studies, but not commercially available, because such studies are too specific and normally employ a particular type of the assay only once. This prompts researchers to develop their own solutions for exploring herd immunity. In this work we discuss theoretical principles and practical approaches to developing multiplex screening assays and give examples of protocols and recommendations based on our own experience in the field.

Keywords: immunological memory, humoral immunity, vaccine-preventable infections, measles, mumps, rubella, hepatitis B, serologic diagnosis, multiplex immunoassay

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✉ **Correspondence should be addressed:** Elena Mazunina
ul. Gamalei, d. 18, Moscow, Russia, 123098; naldinaelena@rambler.ru

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ПРОТОКОЛ РАЗРАБОТКИ СКРИНИНГОВОЙ ТЕСТ-СИСТЕМЫ ДЛЯ ОЦЕНКИ ИММУНОЛОГИЧЕСКОЙ ПАМЯТИ К ВАКЦИНОУПРАВЛЯЕМЫМ ИНФЕКЦИЯМ: ОДНОВРЕМЕННОЕ ОПРЕДЕЛЕНИЕ ТИТРА АНТИТЕЛ К КОРИ, ПАРОТИТУ, КРАСНУХЕ И ГЕПАТИТУ В

Е. П. Мазунина[1] ✉, Д. А. Клейменов[1], В. А. Мануйлов[1,2], В. А. Гущин[1,3], А. П. Ткачук[1]

[1] Лаборатория трансляционной биомедицины,
Национальный исследовательский центр эпидемиологии и микробиологии имени почетного академика Н. Ф. Гамалеи, Москва

[2] Институт молекулярной медицины,
Первый Московский государственный медицинский университет имени И. М. Сеченова (Сеченовский университет), Москва

[3] Кафедра вирусологии, биологический факультет,
Московский государственный университет имени М. В. Ломоносова, Москва

Скрининговые мультиплексные тест-системы для оценки уровня содержания антител к вакциноуправляемым инфекциям активно используются в масштабных сероэпидемиологических исследованиях. Между тем, они отсутствуют в виде коммерчески доступных продуктов, т. к. задачи подобных исследований достаточно узкоспецифичны и ограничены разовым применением таких наборов. В результате исследователи должны самостоятельно разрабатывать и внедрять мультиплексные тест-системы при изучении иммунологической памяти населения. В работе обсуждаются теоретические и практические основы разработки мультиплексных скрининговых тест-систем, приводятся протоколы и рекомендации, основанные на практическом опыте авторов.

Ключевые слова: иммунологическая память, гуморальный иммунитет, вакциноуправляемые инфекции, корь, паротит, краснуха, гепатит В, серодиагностика, мультиплексный иммунный анализ

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✉ **Для корреспонденции:** Мазунина Елена Петровна
ул. Гамалеи, д. 18, г. Москва, 123098; naldinaelena@rambler.ru

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Excellent performance characteristics are crucial for screening assays used in large-scale seroepidemiologic studies. Such assays are designed to screen for antibodies against various vaccine-preventable infections and customized for equipment employed for multiplex suspension analysis. They are cost-effective and time-saving and require smaller sample volumes. However, there are no commercially available multiplex screening assays on the market, ready for use in large-scale seroepidemiologic research normally conducted by laboratories and involving dozens of thousands of samples. There are no

standard lists of antibodies to be measured by such assays, which disrupts their commercial potential, driving labs to develop their own in-house multiplex kits. In turn, this task requires a high level of expertise: lab personnel are expected to master special techniques of screening assay development and validation.

In this work we describe a protocol for the development of a multiplex immunoassay (MIA) to measure antibodies against a few vaccine-preventable infections, namely measles, mumps, rubella and hepatitis B (Fig. 1). We discuss possible problems associated with each stage of the process and offer solutions. The proposed protocol is used in the Laboratory of Translational Biomedicine of Gamaleya National Research Center for Epidemiology and Microbiology, Moscow. We provide detailed description for each step and use the actual data. We hope that this work will be a good guide for our colleagues from other laboratories in the development of multiplex solution-phase multiplex assays, aiding research on herd immunity to vaccine-preventable infections in Russia.

I. Design of the experiment

Protocols for designing and performing the indirect serological bead-based immunoassay were developed based on the recommendations of Luminex (USA), the manufacturer of MagPlex™-C microspheres and the MAGPIX analyzer [1], literature analysis [2–10], including a number of immunoassay manuals [11–13] and validation guides [11, 14–16], and our own experience.

Our method for antibody detection in blood samples relies on the use of capture antigens (AGs) covalently bound to the surface of magnetic beads and detection goat anti-human IgG antibodies conjugated to phycoerythrin. This method has a broader dynamic range, compared to ELISA, and allows simultaneous detection of up to 50 different analytes, if signal detection is performed on MAGPIX. The diagram of the method is shown in Fig. 1.

The schematic of the experiment is shown in Fig. 2.

Our multiplex immunoassay is intended for simultaneous detection of IgG against measles, rubella, mumps and hepatitis B (anti-HBs) in human blood serum. Sample preparation takes 1 hour, including incubations; another 10 minutes are allocated to reagents preparation and washing. With a 96-well plate the analysis takes about 40 min. The whole procedure lasts for less than 2 hours.

II. Preparation of monoplexes

1. Materials

The following components are needed to prepare a monoplex:

– antigens of those pathogens the samples will be tested for (our assay will be used to detect IgG antibodies to measles, mumps, rubella and hepatitis B);

– 4 different regions of magnetic carboxylated microspheres (beads) by MagPlexTM-C;

– serum panels previously characterized using any assay certified in Russia;

– detection antibodies, i.e. PE-conjugated goat anti-human IgG (One Lambda, Thermo Fisher Scientific, USA).

The antigens were selected based on the purpose underlying multiplex assay development. Ultimately, we aimed to estimate population immunity to vaccine-preventable infections. Vaccines against measles, rubella and mumps, registered in Russia, are live vaccines, therefore the priority was given to native antigens. We also used recombinant antigens. Vaccines against hepatitis B contain recombinant surface antigen virus (HBsAg) of the hepatitis B virus of ayw or adw genotypes, therefore, the latter were selected as the 4th component of the multiplex (Table 1).

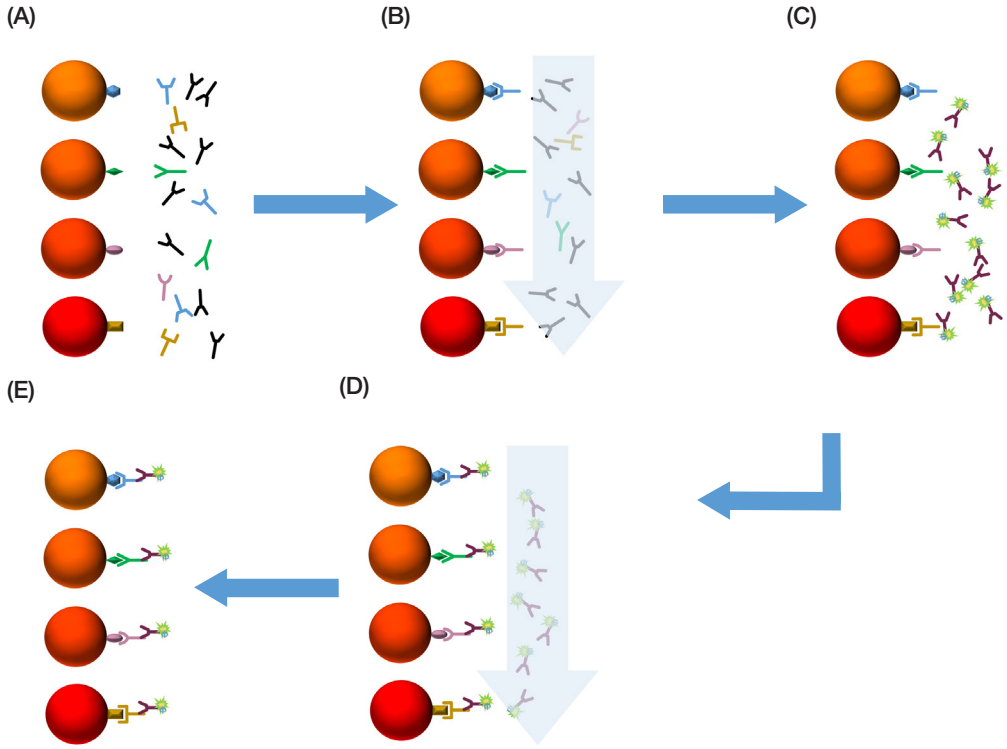

Fig. 1. Schematic representation of indirect serologic multiplex immunoassay, showing 4 bead regions, each bead coupled to a capture antigen. **(A)** Serum is added to the bead suspension; the first incubation is carried out. **(B)** Wash 1: unbound serum components are removed. **(C)** Phycoerythrin-conjugated anti-human detection antibodies are added to the coupled beads and the second incubation takes place. **(D)** Wash 2: unbound components (conjugate) are removed. **(E)** Beads are analyzed on the MAGPIX workstation

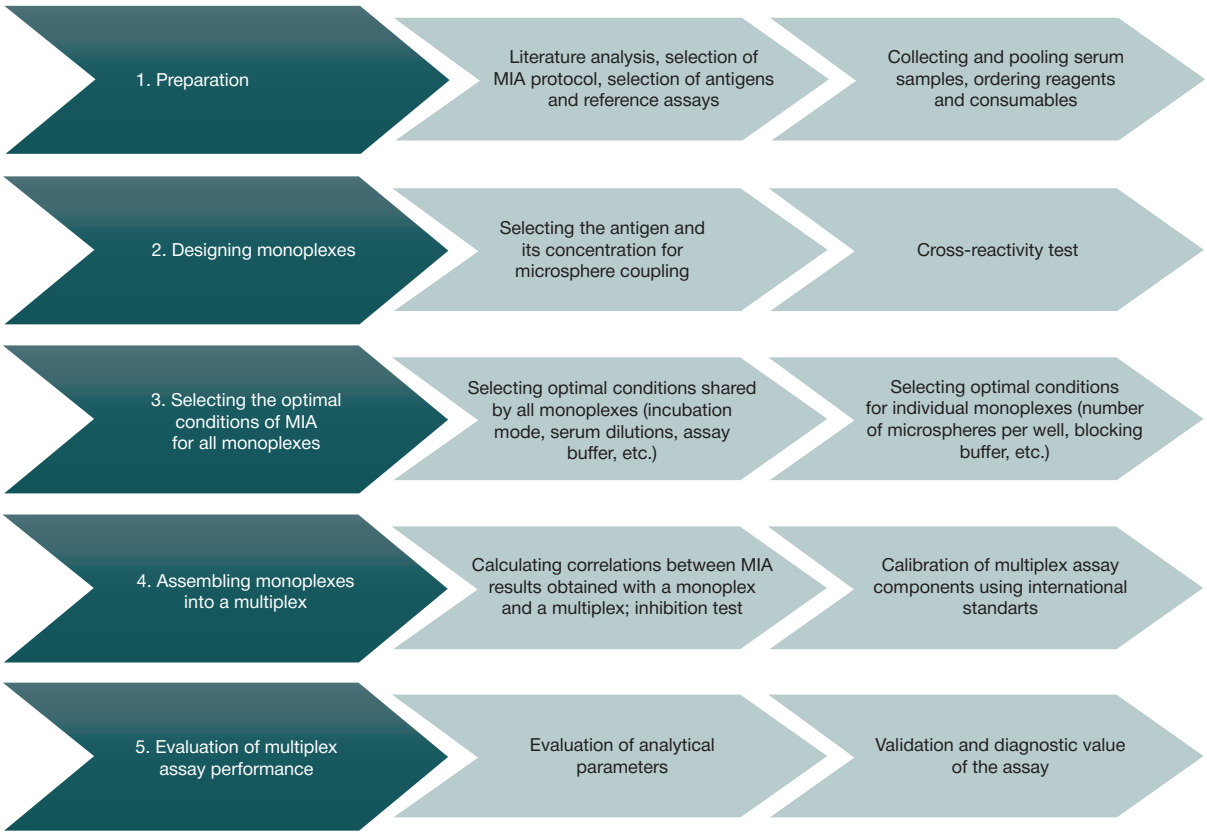

Fig. 2. Diagram showing steps of assay development

For simultaneous detection of IgG against 4 pathogens in a single reaction, we needed a set of 4 corresponding bead regions (MagPlex™-C microspheres). These microscopic beads (6.5 μm in diameter) are the basis of xMAP technology. They are color-coded with 2 to 3 fluorescent dyes, which allows generating up to 500 different regions and, therefore, designing multiplexes for measuring up to 500 analytes in one sample. Magnetic properties of the beads make the protocol simpler: using a magnetic rack for a polystyrene 96-well plate, one can separate the beads from the suspension contained in a well by mere shaking; the microspheres with the captured analyte will stay in the well pelleted by the magnet.

In the course of our experiment we prepared monoplex suspensions of beads conjugated with antigens and also evaluated the resulting multiplex using test samples of human serum. The serum panel must include the samples characterized by a well-established certified method. We used 98 serum samples run through ELISA-based assays (Vector-Best, Russia) and analyzed on automated workstations, including BioPlex (for rubella and measles; Bio-Rad, USA), Architect (for rubella and hepatitis B, Abbott, USA), Liason (for mumps; DiaSorin, Italy) and Immulite (for rubella; Siemens, Germany). To prevent damage to serum samples and to avoid multiple freeze-thaw cycles, all samples were diluted 1 : 2 in preautoclaved 100 % glycerol and stored at –20 ºC.

Microsphere/antigen/IgG complexes were fluorescently visualized using PE-conjugated goat anti-human IgG (detection antibodies), which selectively interacts with human IgG heavy chains.

To measure IgG concentrations in the studied serum samples, expressed in IU/ml, we constructed a calibration curve. Supply of international standards (IS) containing known quantities of immunoglobulins against a particular pathogen is limited; therefore, we used IS for calibration of serum-based secondary standards (SS). For our experiment we ordered WHO International Standards from the collection of the National Institute for Biological Standards and Control (NIBSC, UK) (Table 2). SS were used as an internal standard for the multiplex and as a reference calibration standard for each assay [11]. Because there are no international standards for mumps, we used calibration samples from a commercial ELISA kit. Immunoassay kits should contain human serum for quality control. We used controls from the NIBSC collection (Table 2).

2. Microsphere coupling – obtaining monoplexes

We used two different protocols to couple microspheres to the selected antigens. Both are based on carbodiimide chemistry, but one is a modification of the other. A two-step covalent coupling protocol was used in the case of the native rubella and mumps antigens and the recombinant measles antigen. This method is recommended if the activation molecule is more than 10 kDa in size. Otherwise, as is the case with recombinant HBsAg, the microspheres are modified with a chemical spacer (adipic acid dihydrazide, ADH) in the course of a single-step carmodiimide reaction. In our experiment, following microsphere modification, the capture antigens were coupled by a carbodiimide reaction to the spacer seated on the microsphere surface. It was necessary to “lift” the small peptide over the rough surface of the bead and to facilitate the reaction between the antigen and the analyte.

2.1 Coupling of 10^6 microspheres by a 2-step carbodiimide reaction

All solutions were prepared in distilled water (dH_2O) using the following reagents:

• NaH_2PO_4•H_2O (0.1 M NaH_2PO_4, pH 6.2) for the activation buffer;
• MES hydrate (50 mM MES, pH 5.0) for the binding buffer;
• phosphate buffered saline with 0.02 % Tween-20, 0.1 % BSA, 0.05 % NaN_3, pH 7.4 (below referred to as PBS-TBN) — the wash buffer;
• 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysulfosuccinimide (s-NHS), 50 mg/ml of each reagent. Prepared immediately before use.

Equipment:
• magnetic rack for 1.5 ml Eppendorf tubes;
• ultrasonic bath;
• centrifuge/vortex mixer;
• lab rotator;
• automated cell counter or Goryaev chamber and microscope;
• low-bind 1.5 ml Eppendorf tubes.

Protocol for a two-step carbodiimide conjugation:
– preparation of microspheres
1. mix the flask with the microspheres by rotation for 2 min at 20 rpm;
2. transfer the required amount of microspheres to a clean tube; bring the volume up to 250 µL with dH_2O;
3. vortex/sonicate for 20 s, put the tube in the magnetic rack (below referred to as the magnet). Leave it to sit for 1 min, remove the supernatant;
– microspheres activation:
4. add 80 µL of the activation buffer to the tube, vortex for 20 s;
5. add 10 µL of EDC and s-NHS each, vortex for 20 s;
6. leave it to seat for 20 min, mix by vortexing for 20 s every 10 min;
– wash 1:
7. leave the tube on the magnet for 1 min, remove the supernatant;
8. add 250 µL of the binding buffer to the tube;
9. vortex/sonicate for 20 s, leave on the magnet for 1 min, remove supernatant;
10. repeat steps 8 and 9;
– bead coupling:
11. add to the tube 100 µL of the coupling buffer; vortex/sonicate for 20 s;
12. add the antigen (Table 3);
13. bring the solution volume up to 500 µL by adding the binding buffer;
14. place the tube on the rotator for 120 min at 20 rpm, protected from the light;
– wash 2:
15. leave the tube on the magnet for 1 min; remove the supernatant;
16. add to the tube 1000 µL of the wash buffer;
17. vortex/sonicate for 20 s; magnet — 1 min; remove the supernatant;
18. repeat steps 16 and 17 twice;
– finishing the procedure:
19. resuspend the microspheres in 1000 µL of the storage buffer (PBS-TBN or any other blocking buffer, selected experimentally);

Table 1. Antigen candidates for the assay

Infection	Type	Trade name	Manufacturer
Hepatitis B	Recombinant	Hepatitis B virus, subtype ayw	Bialexa, Russia
		Hepatitis B virus, subtype adw	
Rubella	Recombinant	Rubella virus E1 protein	Bialexa, Russia
		Rubella virus E2 protein	
		Rubella virus C protein	
	Native	Rubella K1S grade antigen	Microbix Biosystems, Canada
		Rubella K2S grade antigen	
		Rubella virus grade 2 antigen	Jena Bioscience, Germany
Measles	Recombinant	Measles virus nucleocapsid protein NCP	Kapel Biotech Company, Russia
	Native	Measles grade 2 antigen	Microbix Biosystems, Canada
		Measles virus antigen (Premium)	Jena Bioscience, Germany
		Measles virus antigen	
Mumps	Recombinant	Mumps virus nucleocapsid protein NCP	Kapel Biotech Company, Russia
	Native	Mumps grade 2 antigen	Microbix Biosystems, Canada
		Mumps/Parotitis virus antigen	Jena Bioscience, Germany

Table 2. Standards and serum controls used in the development of the multiplex assay

Infection	Standard/control name	Concentration or number of antibodies	Source
Hepatitis B	WHO International Standard Second International Standard for anti-hepatitis B surface antigen (anti-HBs) immunoglobulin, human	100 IU in a vial	NIBSC
	Anti-Hepatitis B Surface Antigen Quality Control Serum 1	219.0±16 IU/ml	NIBSC
Rubella	WHO International Standard Anti Rubella Immunoglobulin, Human	1600 IU in a vial	NIBSC
	Anti-Rubella Quality Control Reagent Sample 1	25.5±2,9 IU/ml	NIBSC
Measles	WHO International Standard 3rd International Standard for Anti-Measles	3 IU in a vial	NIBSC
	Anti-Measles Quality Control Reagent Sample 1	754.6 mIU/ml	NIBSC
Mumps	Anti-Mumps Quality Control Reagent Sample 1	728.6 EU/ml	NIBSC

20. measure bead concentrations in the cell counter or Goryaev chamber. Calculate the required amount of beads for MIA, accordingly;

21. store the coupled beads in the dark at +4 ºC for 16 h until further analysis (performed on the next day).

*2.2 Coupling of 1.5*10^6 microspheres by two one-step carbodiimide reactions using a spacer*

All necessary solutions were prepared in dH_2O using the following reagents:

- MES hydrate for the binding buffer (0.1 M MES, pH 6.0);
- MES hydrate for the wash buffer 1 (0.1 M MES, pH 4.5);
- phosphate buffered saline with 0.02 % Tween-20, 0.1 % BSA, 0.05 % NaN_3, pH 7.4 (below referred to as PBS-TBN) — the wash buffer 2;
- 100 mg/ml ADH (spacer) solution in the binding buffer;
- 100 mg/ml EDC solution in dH_2O. For (7) and (19) the solutions need to be prepared separately.

Equipment:

- magnetic rack for 1.5 ml Eppendorf tubes;
- ultrasonic bath;
- centrifuge/vortex mixer;
- lab rotator;
- automated cell counter of Goryaev chamber and microscope;
- low-bind 1.5 ml Eppendorf tubes.

Protocol for two one-step carbodiimide conjugations:

– preparation of microspheres:

1. mix the flask with microspheres by rotation for 2 min at 20 rpm;

2. transfer the required amount of microspheres to a clean tube, bring the volume up to 250 µL with dH_2O;

3. vortex/sonicate for 20 s, put the plate on the magnet; leave it to sit for 1 min, remove the supernatant.

4. add 500 µL of the binding buffer to the tube;

5. vortex/sonicate for 20 s; leave on the magnet for 1 min; remove the supernatant;

– coupling a spacer to beads:

6. add to the tube 30 µL of ADH (1mg per 500,000 beads);

7. add to the tube 30 µL of EDC (1mg per 500,000 beads);

8. bring the volume up to 500 µL with the binding buffer;

9. place the tube on the rotator for 60 min at 20 rpm, protected from the light;

– wash 1:

10. magnet – 1 min; remove the supernatant;

11. add 500 µL of the wash buffer 1 to the tube;

12. magnet – 1 min; remove the supernatant;

13. repeat steps 11 and 12 two more times;

– finishing spacer coupling:

14. resuspend microspheres in 1,000 µL of the wash buffer 1;

15. measure bead concentrations in the cell counter or Goryaev chamber. Calculate the required amount of beads for MIA, accordingly;

– coupling antigens with spacer on beads:

16. vortex/sonicate for 20 s; place on the magnet for 1 min; remove the supernatant;

17. add 100 µL of the binding buffer; vortex/sonicate for 20 s;

18. add the required amount of the peptide (Table 3);

19. add 30 µL of EDC (1 mg per 500,000 microbeads);

20. bring the volume up to 500 µL with the binding buffer;

21. mix by rotation for 120 min at 20 rpm, protected from the light;

– wash 2:

22. place the tube on the magnet. Leave for 1 min; remove the supernatant;

23. add 1,000 µL of the wash buffer 2;

24. vortex/sonicate for 20 s; magnet — 1 min; remove the supernatant;

25. repeat steps 23 and 24 two more times;

– finishing the procedure:

26. resuspend the microspheres in 1,000 µL of the storage buffer (PBS-TBN or any other blocking buffer, selected experimentally). For the hepatitis B monoplex we used PBS-TBN;

27. measure bead concentrations in the cell counter or Goryaev chamber. Calculate the required amount of beads for MIA, accordingly;

28. store the coupled beads in the dark at +4 ºC for 16 h for further analysis (performed on the next day).

3. Protocol for multiplexed indirect (serological) immunoassay

Before validating the performance of the multiplex, it is important to evaluate each monoplex component separately and optimize conditions for all monoplexes. This procedure is referred to as *Selecting the optimal conditions of MIA for all monoplexes* in Fig. 2. Evaluation is based on the analysis of serum samples using the following protocol.

Materials:

- a 96-well polystyrene plate (below referred to as the plate);
- 0.5 ml, 1.5 ml and 2 ml test tubes;
- a set of serum samples previously tested for the presence of target IgG;
- suspensions of ready-for-use microspheres conjugated with antigens;
- a 100X stock solution of phycoerythrin-conjugated detection antibodies;
- PBS-TBN.

Equipment:

- plate magnet (Magnetic Plate Separator, Luminex);
- centrifuge/vortex mixer;
- thermoshaker;
- ultrasonic bath;
- MAGPIX analyzer (Luminex).

The protocol:

1. preheat the thermoshaker to +37 ºC and switch on MAGPIX;

2. in microtubes, prepare 1 : 50 dilutions of serum samples using PBS-TBN;

3. prepare a 50 µL suspension of microspheres (2,500 beads per well/reaction):

- vortex/sonicate for 20 s,
- collect the required volume of the suspension to a new tube; adjust the transferred volume with PBS-TBN;

4. add 50 µL of the suspension and 50 µL of the diluted serum to each plate well. The final serum dilution will be 1 : 100;

5. place the plate in the thermoshaker and incubate for 30 min at +37 ºC by mixing at 800 rpm;

6. while the samples are incubating, prepare a solution of detection antibodies. Take the required volume of the stock antibody solution and dilute it 1 : 100 with PBS-TBN (you will need 50 µL of the final solution per well; final antibodies concentration should be 2.5 µg/ml per well);

7. remove the plate from the thermoshaker and leave it on the magnet for 2 min; then separate the liquid without removing the plate from the magnet;

8. wash the samples with PBS-TBN: add 100 µL PBS-TBN to each well using the multichannel pipette, mix in the

thermoshaker for 30 s, place the plate on the magnet for 2 min, remove the liquid, wash again;

9. resuspend the microspheres in 50 µL PBS-TBN per well;

10. add 50 µL of the prepared solution of detection antibodies to each well (see step 6);

11. second incubation: incubate for 30 min at +37 ºC: place the plate in the thermoshaker and mix at 800 rpm for 30 min;

12. wash with PBS-TBN (see step 8);

13. after washing, add 100 µL PBS-TBN to each well, mix in the thermoshaker for 30 s;

14. load the plate into MAGPIX and run the analysis.

Importantly, such parameters as temperature, incubation time, serum dilution, or a concentration of detection antibodies are variable, and these variations can affect fluorescence intensity (expressed in MFI units). Adjusting these parameters so that they would be equally beneficial for the performance of each monoplex is what optimization is about.

4. Optimization of monoplexes

The importance of the optimization step in the development of a multiplex assay cannot be overrated. Optimization is a search for the best conditions for each monoplex ensuring that the following requirements are met:

- low MFI values for the negative control (for example, rabbit serum or human serum free from immunoglobulins);
- the widest possible range of signal intensities in the serum samples with and without the studied antibodies;
- the results obtained using monoplexes are expected to be consistent with the results obtained using reference ELISA.

Optimization covers a wide range of parameters, including temperature and incubation time, serum dilution and a solution for serum dilution, or concentrations of detection antibodies, affecting the performance of all 4 monoplex components. But a few other factors also need to be optimized specific for each monoplex, such as a blocking buffer, antigen concentrations for coupling, and the number of beads in the monoplex per well. These parameters were accounted for, and the optimal conditions were selected for all monoplex components of the assay (Table 3).

Because some of the selected antigens can be recombinant (obtained through expression in *Escherichia coli*), target antigen preparations may be contaminated with the antigens of this bacterium. Antibodies to *E. coli* that are likely to be present in the studied serum sample may produce a false positive signal affecting the total MFI. To avoid this effect, we added the *E. coli* lysate (3 %) to the serum dilution buffer to bind *E. coli*-specific immunoglobulins [5].

Optimization also includes evaluation of cross-reactivity for each monoplex; the earlier it is performed, the better. Cross-reactivity tests show how specific is, for example, the monoplex with the measles virus antigen for IgG antibodies to the measles virus and whether it interacts with antibodies to other infections.

The cross-reactivity test is performed according to the protocol described above under the conditions presented in Table 3. Thus, the monoplex component of the assay containing beads conjugated to the measles antigen is tested using serum without IgG antibodies to measles but with IgG antibodies to other pathogens. Other monoplexes are tested in a similar manner. If MFI value is at the limit of detection (LOD), the monoplex is not cross-reactive; otherwise the capture antigen needs to be replaced.

At this stage of our experiment, we tested the optimized monoplexes designed to detect IgG antibodies to measles, rubella and mumps using a panel of 70 serum samples. Conditions are shown in Table 3. The results for MIA are presented in Fig. 3–5, expressed in MFI, because calibration was yet to be implemented, and the assay could not yet be used for quantification. ELISA kits (Vecto-Rubella-IgG, Vecto-Measles-IgG, Vecto-Mumps-IgG by Vector-Best) were used as reference.

The rubella monoplex successfully differentiated between serum samples with and without IgG antibodies to rubella; the results were consistent with those obtained by ELISA (Fig. 3). However, measles and mumps monoplexes were found to require further optimization, because they generated high MFI for serum samples that did not contain IgG antibodies (the values were close to or above the grey zone values, see Fig. 4, 5).

Clearly, without calibration we cannot compare our monoplexes and ELISA, but we still can estimate the performance of each monoplex and decide whether further optimization is required and the monoplex is ready for calibration.

Table 3. Optimal conditions for the developed immunoassay

Parameter		Monoplex			
Virus		Measles	Mumps	Rubella	Hepatitis B
Antigen	Name	Measles virus nucleocapsid protein NCP	Mumps/Parotitis virus antigen (native)	Rubella K2S grade antigen (native)	Recombinant HbsAg, subtypes ayw and adw
	Concentration, µg per million beads	5	10	10	20 (10 HbsAg ayw + 10 HbsAg adw)
PBS-TBN		PBS-TBN			
Serum dilution, dilution buffer		1 : 100, PBS-TBN + *E. coli* lysate			
Concentration of detection antibodies		2.5 µg /ml			
Incubation		• first incubation — 30 min, second incubation — 30 min • incubation temperature — +37 °C • mixing by rotation at 800 rpm			
Number of beads per well for each monoplex		2 500			

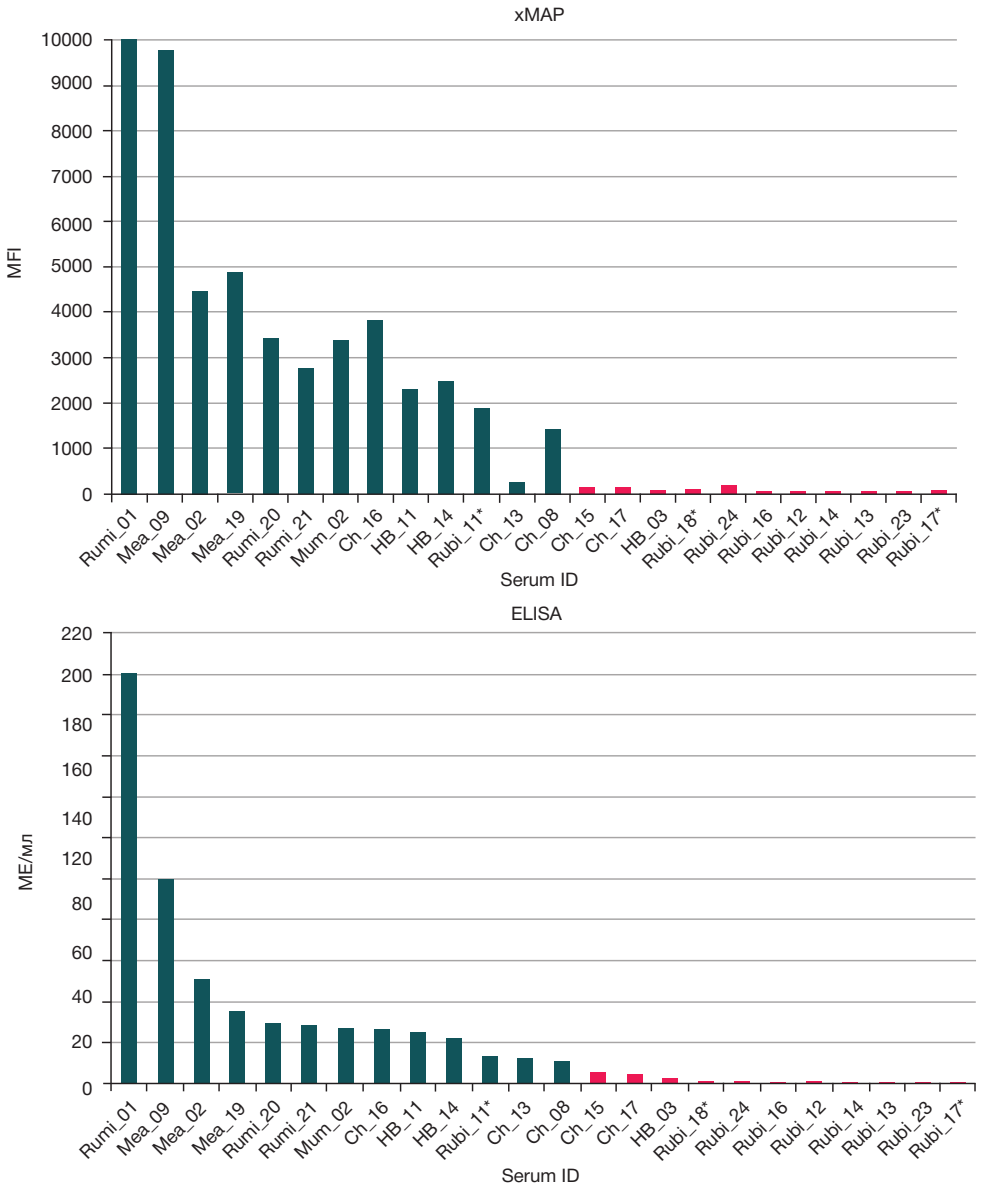

Fig. 3. Levels of IgG to rubella in human serum measured by xMAP and ELISA

Pooled serum samples were pretested in the reactions with monoplexes in a series of fourfold dilutions from 1 : 100 to 1 : 409,600. The resulting calibration curves were used to estimate whether serum pools could be used as secondary standards (Fig. 6).

III. From monoplex to multiplex

After each monoplex has been optimized, pretested using the serum panel and results have been compared to ELISA, the protocol is selected; in the next step monoplexes are combined into a multiplex and the results obtained using the multiplex and its monoplex components are compared. These tests are run in parallel using the serum panel [4]. Based on the results, correlations are established. At the moment, we are comparing qualitative characteristics of the multiplex for the detection of IgG antibodies to measles, rubella, mumps and hepatitis B and its monoplex components. Below we briefly describe the validation procedure for the multiplex.

When talking about optimization, we mentioned specificity of the assay. It should be evaluated in the optimization step (see 3 in Fig. 2). However, at this stage specificity and cross-reactivity should not be confused. Specificity is evaluated using homologous and heterologous inhibition of serum samples by antigens [3, 7–11]. For the inhibition test, a serum sample is needed with a known high concentration of IgG antibodies to all 4 pathogens. The sample is divided into 4 aliquots; each aliquot is incubated with one of 4 antigens. After preincubation, the multiplex is added to the serum.

The control (non-preincubated serum) is also included in the reaction. Homologous inhibition demonstrates specificity of a monoplex component of the multiplex assay for the homologous antigen, while heterologous inhibition indicates a nonspecific reaction between a monoplex and the heterologous antigen. Thresholds for homologous and heterologous inhibitions are 80–120 % and < 30 %, respectively. If the assay has passed the specificity test, standardization should be performed next, i.e. calibration using international standards (Table 2). First, serum samples should be selected with high concentrations of IgG antibodies against each pathogen; the samples are then pooled into a positive pool (which could be later used as the secondary standard). Then titration is performed of the international standard and the serum pool in a single run using a fourfold series of dilutions starting from 1 : 100 [11],

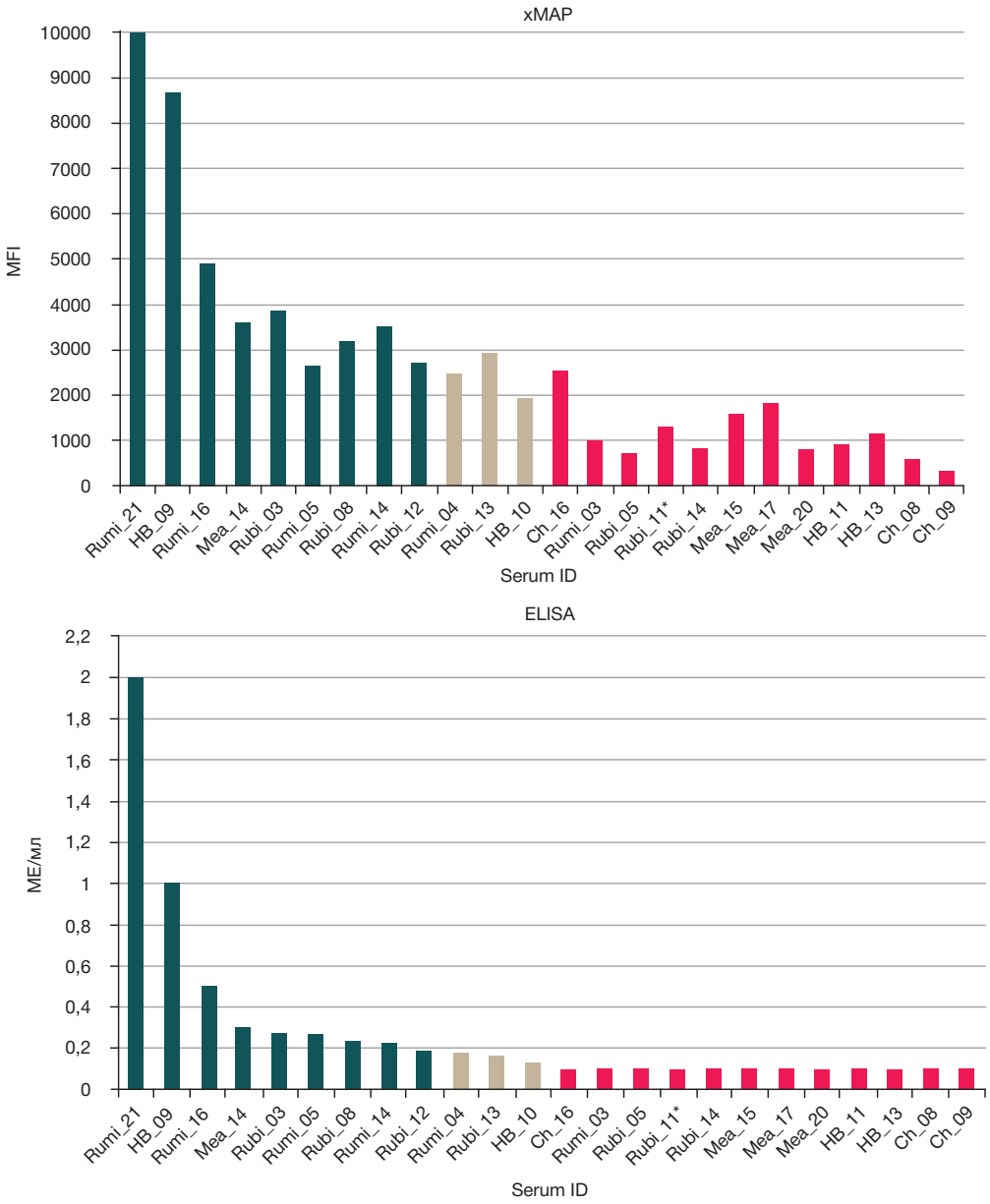

Fig. 4. Levels of IgG to measles in human serum measured by xMAP and ELISA

in each of 4 monoplexes according to the protocol, under the selected conditions. Based on the results, calibration curves are constructed using the predetermined values of international standards (expressed in IU/ml) and the values of the same standards obtained in the course of the experiment expressed in MFI for each monoplex. Calibration curves are used to determine concentrations of each analyte in the positive serum pool; then titration curves are constructed and conclusions are made whether this pool can be used as the secondary standard or it should be diluted.

Standardization of the multiplex is carried out following the same procedure (IS and SS titration in parallel, in the multiplex suspension). Multiplex calibration curves are compared with those for monoplexes, obtained in the previous step, and correlations are drawn. Then IS and SS titration curves are compared, and the decision is made whether the SS titration curve can be used as a calibration curve for the multiplex.

Then the optimal mathematical model is selected to accurately describe the obtained calibration curve and to calculate antibodies concentrations (MIA) expressed in international units.

IV. Analytical parameters, validation and diagnostic value of a screening assay

Validation is a series of experiments performed to reliably assess analytical parameters of the assay using a certified analytical method. Validation aims to test the feasibility of the developed assay and determine its limitations that may be critical for routine use. The assay has a lot of analytical parameters; some of them are analyzed and improved at the start of the experiment (for example, cross-reactivity). Any experiment involving serum samples with known analyte concentrations (detected by a certified technique) is, in its essence, a part of the validation process. It is impossible to say when the development of the assay ends and validation begins.

Below we describe basic analytical characteristics of the assay and propose a validation plan.

Sensitivity is in the broad sense of this word an ability to detect an analyte. It can be expressed as a minimal concentration of this analyte reliably detected by the assay. Analytical sensitivity is evaluated by running the zero sample without the analyte through the assay; the mean value of the results plus 2 to 3

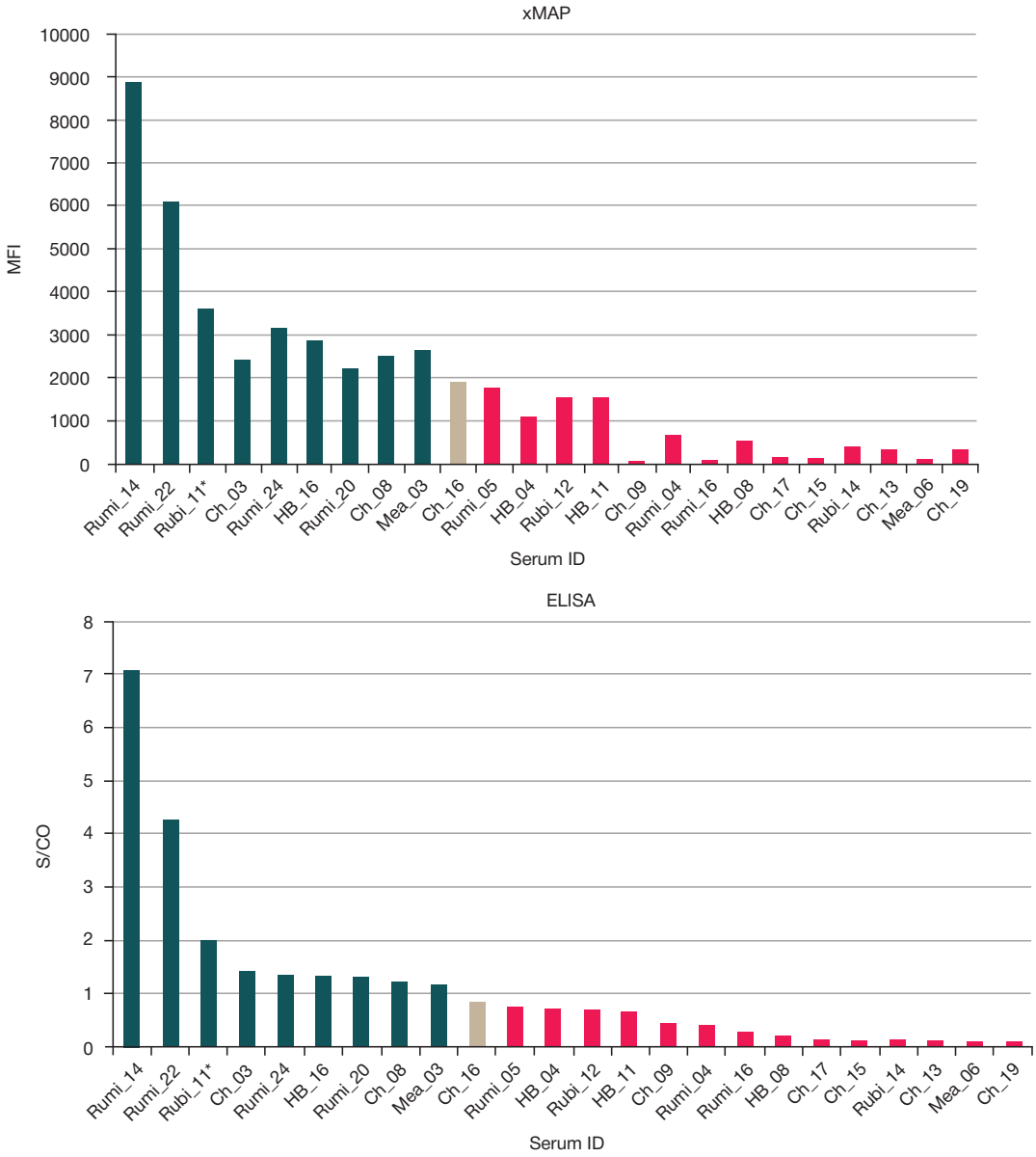

Fig. 5. Levels of IgG to mumps in human serum measured by xMAP and ELISA

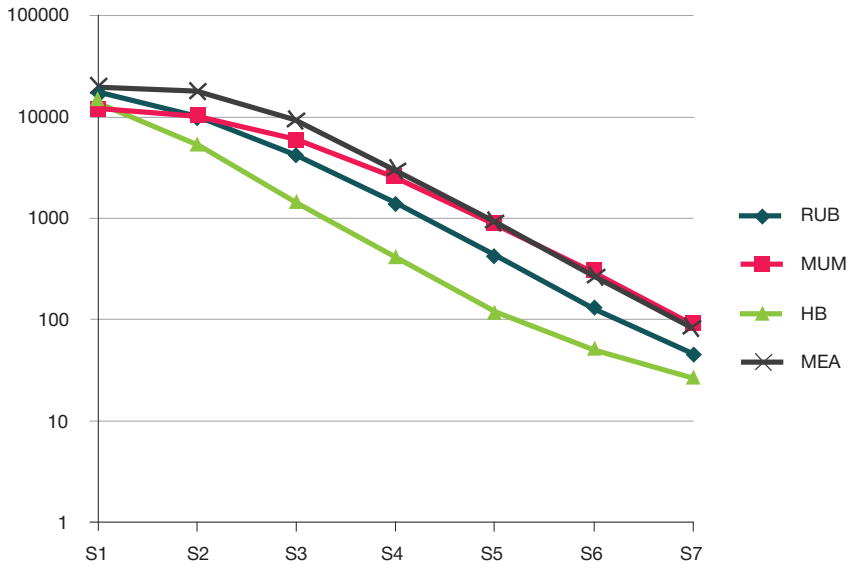

Fig. 6. Curves for serum pools dilutions with high antibody titers to rubella (RUB), measles (MEA), mumps (MUM) and hepatitis B (HB)

standard deviations are the analytical sensitivity. Another term for it is limit of detection (LOD). In other words, it is a minimal concentration of the analyte reliably detected (but no necessarily quantitatively) by the studied assay. ULOQ and LLOQ are upper and lower limits of quantification, the maximal and minimal analyte quantities that can be detected at certain accuracy and reproducibility values. Another type of sensitivity is functional sensitivity- a minimal analyte concentration detected in a series of runs with the coefficient of variance below a certain threshold (10–20 %).

Precision is the variability of the results obtained in a series of experiments with the same sample run through the assay under identical conditions; it is expressed as a coefficient of variance (CV, %). There are different types of precision. Within-run precision (intra-assay variation) should be < 10 %. Between-run precision (inter-assay variation) is expected to be < 20 %. Other types of precision include variations between reagents, equipment and even researchers. Reproducibility refers to the precision of the results obtained with the same technique but it different laboratories.

Specificity is an ability of the antibody to react with only one particular antigen (one of the antigen/antibody pair is the studied analyte). Cross-reactivity describes the extent of antibody ability to react with other substances besides the analyte/antigen.

Accuracy describes how consistent is the result obtained using the studied assay with the actual value of the measured parameter. Accuracy is crucial for all quantitative assays. It is determined by running a sample with a known quantity of the analyte through the assay. The ratio of the anticipated value to the measured one shows the degree of recovery.

Linearity is an ability of the assay to produce results directly proportional to the analyte concentration in the sample. To estimate linearity, a series of standard dilutions of the analyte is prepared, at concentrations ranging from 50 % to 130 % by diluting the original solution. The concentrations are then measured using the studied method. Based on the results the calibration curve is constructed representing the linear dependence between the calculated concentrations and the original concentrations (in normalized coordinates). To estimate linearity, r^2 correlation coefficient, the slops and the y-intercept are calculated.

The assay range is an interval between the maximal (ULOQ) and the minimal (LLoQ) analyte concentrations in the analyzed sample, for which the studied method has an acceptable level of precision, accuracy and linearity.

Apart from analytical characteristics, there are a few diagnostic parameters used to estimate the diagnostic value of an assay. They are analyzed in the course of assay development and compared to the results obtained using the reference method. Those include diagnostic sensitivity (frequency of analyte detection in the samples in comparison with the reference method; expressed in %); diagnostic specificity (frequency of the negative result, i.e. the absence of the analyte in the sample previously tested by a reference method; expressed in %), and some other.

All the parameters mentioned above can be assessed during the validation step. Fig. 7 shows a diagram representing a single assay run that can yield data on almost all assay parameters. Rows 1–3 and 10–12 of the plate contain calibrators in the standard dilution buffer (PBS-TBN). Eight dilutions of calibration samples are obtained in 6 replicates. These samples will provide information about the calibration curve, intra-assay variation, and the upper and lower limits of detection. Wells marked by B (blank wells) contain only the diluent (PBS-TBN) without the analyte. The mean value for these 12 wells will be used to determine the background signal, the limit of detection. Rows 4–6 C–H contain samples with the analyte diluted in the standard dilution buffer (PBS-TBN) in 3 replicates (S+B = sample+buffer). Rows 7–9 C-H contain samples with the analyte diluted in the original matrix (rabbit serum or human serum free of antibodies) (S+M = sample+matrix). The obtained data can be used to estimate accuracy, recovery from the diluent, linearity and intra-assay variation. To evaluate inter-assay variation, a few similar runs should be performed. Specificity is assessed by running cross-reactivity and inhibition tests described above.

Upon validation we plan to determine the diagnostic value of the assay and compare it to that of a commercial ELISA. We plan to analyze at least 400 serum samples: 100 per pathogen.

CONCLUSION

The xMAP technology has been recognized as a rapid, sensitive and accurate method for indirect serologic immunoassay. Multiplex immunoassays have demonstrated their efficiency in serological studies of herd immunity against vaccine-

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	S1	B	B	B	B	B	B	S1	S1	S1
B	S2	S2	S2	B	B	B	B	B	B	S2	S2	S2
C	S3	S3	S3	S+B (1)	S+B (1)	S+B (1)	S+M (1)	S+M (1)	S+M (1)	S3	S3	S3
D	S4	S4	S4	S+B (2)	S+B (2)	S+B (2)	S+M (2)	S+M (2)	S+M (2)	S4	S4	S4
E	S5	S5	S5	S+B (3)	S+B (3)	S+B (3)	S+M (3)	S+M (3)	S+M (3)	S5	S5	S5
F	S6	S6	S6	S+B (4)	S+B (4)	S+B (4)	S+M (4)	S+M (4)	S+M (4)	S6	S6	S6
G	S7	S7	S7	S+B (5)	S+B (5)	S+B (5)	S+M (5)	S+M (5)	S+M (5)	S7	S7	S7
H	S8	S8	S8	S+B (6)	S+B (6)	S+B (6)	S+M (6)	S+M (6)	S+M (6)	S8	S8	S8

Fig. 7. Diagram illustrating the validation process of the studied assay. S1–8 — wells used to analyze calibrators and construct the calibration curve. B — wells containing a standard diluent; S+B — samples with the analyte diluted in a standard dilution buffer; S+M — samples with the analyte diluted in the matrix (rabbit serum or human serum free of antibodies)

preventable infections. They can simultaneously detect a number of different analytes, are fast and have excellent analytical characteristics. We hope that our tetraplex will aid large-scale seroepidemiological studies of herd immunity against vaccine-preventable measles, rubella, mumps and hepatitis B. The proposed protocol can be used for the development of other assays aimed to measure antibody titers against other vaccine-preventable and significant pathogens.

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ESTIMATING THE ACTUAL SUSCEPTIBILITY OF DECREED POPULATION TO MEASLES, RUBELLA AND MUMPS

Nozdracheva AV ✉, Gritsik AA, Kuzin SN, Semenenko TA

Department of Epidemiology,
Gamaleya Federal Research Center for Epidemiology and Microbiology, Moscow, Russia

As the modern society becomes more vaccine-dependent, the need arises for population immunity studies aimed to estimate the prevalence of antibodies against a particular infection in the donors from different populations, including sentinel groups. This work reveals the actual susceptibility of the Russian population to a number of vaccine preventable infections, including measles, rubella, and mumps. The study was conducted using blood serum samples provided by the Blood serum bank of Gamaleya Federal Research Center for Epidemiology and Microbiology (Moscow). Of 866 samples used in the study, 293 and 117 samples had been collected from healthcare workers in 2011 and 2017, respectively; 220 samples had been obtained from military servants in 2016–2017, and 236 samples had been collected from healthy donors in 2016. All samples were studied by solid phase enzyme immunoassay using diagnostic kits by Vector-Best (Russia). We discovered that 19.4 % and 28.8 % of individuals were susceptible to measles and mumps, respectively, which is insufficient for ensuring epidemiological safety (the herd immunity thresholds for these diseases are 7 % and 15 %, respectively) and puts the population at risk of infection spread, should the pathogens enter the country. The proportion of individuals susceptible to rubella was 6.5 %, which is below the herd immunity threshold (7 %).

Keywords: preventive vaccination, herd immunity, serological surveillance, measles, rubella, mumps, military servants, healthcare workers

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✉ **Correspondence should be addressed:** Anna Nozdracheva
ul. Gamalei, d. 18, Moscow, Russia, 123098; nozdracheva0506@gmail.com

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ОЦЕНКА ФАКТИЧЕСКОЙ ВОСПРИИМЧИВОСТИ ОТДЕЛЬНЫХ ГРУПП ДЕКРЕТИРОВАННОГО НАСЕЛЕНИЯ К ВИРУСАМ КОРИ, КРАСНУХИ И ЭПИДЕМИЧЕСКОГО ПАРОТИТА

А. В. Ноздрачева ✉, А. А. Грицик, С. Н. Кузин, Т. А. Семененко

Отдел эпидемиологии,
Национальный исследовательский центр эпидемиологии и микробиологии имени почетного академика Н. Ф. Гамалеи, Москва

В условиях формирования «вакцинозависимости» современного общества приоритетным является изучение популяционного иммунитета, основанное на оценке распространенности антител к той или иной инфекции в крови доноров из различных, в том числе индикаторных, групп населения. В работе представлены данные о фактической восприимчивости россиян к таким вакциноуправляемым инфекциям, как корь, краснуха и эпидемический паротит. В качестве материала для исследования использовали образцы сывороток крови из Банка сывороток крови Национального исследовательского центра эпидемиологии и микробиологии имени почетного академика Н. Ф. Гамалеи (Москва): 866 образцов, в том числе 293 и 117 образцов от медицинских работников, полученных в 2011 г. и 2017 г. соответственно, 220 образцов от военнослужащих, полученных в 2016–2017 гг., и 236 образцов от условно здоровых доноров, полученных в 2016 г. Все образцы были исследованы методом твердофазного иммуноферментного анализа с использованием предназначенных для изучаемых инфекций тест-систем компании «Вектор-Бест» (Россия). Была выявлена значительная доля восприимчивых к кори (в среднем — 19,4 %) и эпидемическому паротиту (в среднем — 28,8 %) лиц, что не соответствует критерию эпидемического благополучия для этих инфекций (7 и 15 % соответственно) и создает условия для распространения инфекций в случае попадания возбудителей на территорию страны. Доля восприимчивых к краснухе людей составила в среднем 6,5 %, что отвечает критерию эпидемического благополучия (7 %).

Ключевые слова: вакцинопрофилактика, популяционный иммунитет, серологический мониторинг, корь, краснуха, эпидемический паротит, военнослужащие, медицинские работники

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✉ **Для корреспонденции:** Ноздрачева Анна Валерьевна
ул. Гамалеи, д. 18, г. Москва, 123098; nozdracheva0506@gmail.com

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World Health Organization (WHO) estimates that on average 2 billion people suffer from infections every year; 17 million of them die [1]. Vaccination is the most effective and economically sound tool for controlling infections, given that it is promoted worldwide and the involved healthcare workers are committed to its goals. Due to vaccination, approximately 2 to 3 million infection-related deaths were prevented in 2016; but better coverage could have saved another 1.5 million lives [1].

Good vaccination coverage is one of the key indicators of a successful vaccination strategy. To ensure protection against the majority of vaccine-preventable infections, WHO expects vaccination coverage to be at least 95 % among children and 90 % among adults. Officially [2], Russia has already achieved these high goals and now only maintains the recommended rates. Plummeting incidence and mortality related to vaccine-preventable infections indicate that the chosen vaccination strategy is effective.

Inspiring results of vaccination drove WHO to announce a new era in healthcare: smallpox had been eliminated by 1980, no incident cases of polio have been reported in Russia since 2002. Although measles and rubella had not been eradicated by 2010, as planned, there is hope for this to happen in the future. In 2000, the USA declared elimination of measles [3]; however, in 2010 the situation changed: measles incidence rates started to increase in both Americas, Europe, Africa, and the former Soviet Union, including Russia [4]. Therefore, the deadline for measles elimination was extended to 2015 and then beyond 2020.

Growing measles incidence was alarming, prompting the medical community to question the reliability of key indicators of vaccination effectiveness. A few new factors were discovered negatively affecting vaccination outcome, including weak cold chains, unsubstantiated contraindications, increasing vaccine hesitancy among parents and failure to adhere to vaccination schedules. Unfortunately, vaccine hesitancy is becoming a pressing issue in Russia. Polibin et al. [5] indicate that in Russia the population tends to avoid any preventive measures, including vaccination. Parent surveys show that only 80 % of infants below 2 years of age are vaccinated.

Many authors believe that the troubling situation with measles resulted from reduced herd immunity, i. e. the proportion of people susceptible to infection [6–8]. The only way to measure it is to resort to serological surveillance [8, 9]. The proportion of seronegative (or susceptible) individuals is calculated using laboratory serum tests. Seronegativity rates of < 7 % and 15 % for measles and mumps, respectively, are epidemiological health thresholds.

In Russia, serological surveillance is not perfect. One of its downsides is the inability to compare results of studies of serum samples collected in different years, accounting for the rapid evolution of laboratory diagnostic techniques and standards. Besides, serological studies ignore professional occupation of the participants (for example, vaccination requirements for decreed population groups are stricter) and their general health (infections affect humoral immunity). Serum banks may provide a solution here.

A number of Russian researchers argue that healthcare workers contribute to the risk of infection outbreaks in healthcare facilities [10, 11]. Healthcare personnel is regarded by the law as a decreed population group; members of this group must be vaccinated against measles before turning 55 (or 35, in the case of rubella and mumps) [12]. These people are not only at risk of getting infected; they also indirectly signal the epidemiological status of the population.

This study aimed to evaluate the actual susceptibility of the Russian population to measles, rubella and mumps by analyzing the samples from the Serum bank of N. F. Gamaleya Federal Research Centre for Epidemiology and Microbiology, Moscow.

METHODS

We studied 866 serum samples, including:

– 293 samples collected in 2011 from healthcare workers residing in the Central Federal District (CFD);

– 117 samples collected in 2017 from healthcare workers residing in CFD;

– 220 samples collected in 2016–2017 from contracted soldiers (military recruits) serving in Moscow region;

– 236 samples collected in 2016 from seemingly healthy individuals residing in Moscow and Moscow region.

In 2011 the situation with measles significantly deteriorated in Russia, and in 2017 extra preventive measures were taken supported by the State, including immunization of previously unvaccinated population groups and increasing the vaccination age for medical workers from 35 to 55 years [8, 12]. Outbreaks of measles, rubella and mumps are, however, still reported in the armed forces, demonstrating a problem of a closed susceptible group [13].

In our study, vaccination histories of sample donors were unknown, no documented evidence on their vaccination status was available.

The study was designed and conducted in compliance with the guidelines of the Russian Federal Service for Surveillance on Consumer Rights Protection and Human Wellbeing [9]. Serum samples were studied using ELISA and a number of testing kits, including Vecto-Measles IgG, Vecto-Rubella IgG, Vecto-Mumps IgG (by Vector-Best, Russia), according to the manufacturer's protocol.

Statistical analysis was performed in Microsoft Excel and Statistica 6.0 (StatSoft, USA). Significance of differences between the groups was calculated using the chi squared test and Yates' correction. Mean values (m) were calculated for all studied parameters; a 95 % confidence interval was calculated using the Clopper–Pearson method.

RESULTS

In the course of our study, we evaluated immunity to vaccine-preventable infections among different population groups. Results are shown in Table 1.

The table shows that all studied groups were protected against rubella: the proportion of seronegative samples did not exceed the 7 % threshold.

A considerable proportion of study participants were susceptible to measles, military recruits being the most vulnerable group, where the proportion of seronegative samples reached the maximum of 25.5 %, which is 3.6 times higher than the epidemic threshold. Among medical workers, susceptible individuals accounted for 7.7 %.

Among the healthy donors residing in Moscow and Moscow region, the proportion of susceptible individuals was quite large (21.2 %). This people may contribute to the spread of measles, forming epidemic foci, especially in the armed forces, should the pathogen find its way into the region. Thus, the current situation with measles in Moscow and Moscow region can be characterized as unstable.

Table 1. Immunity to measles, rubella and mumps in different population groups residing in Russia (2016–2017)

Population groups	Seronegativity to measles virus, % [CI of 95 %]	Seronegativity to rubella virus, % [CI of 95 %]	Seronegativity to mumps virus, % [CI of 95 %]
Healthcare workers	7.7* [3.6; 14.1]	5.1 [1.9; 10.8]	20.5 [13.6; 29.0]
Military recruits	25.5 [19.8; 31.8]	4.6 [2.2; 8.2]	29.6 [23.6; 36.1]
Healthy individuals	21.2 [16.2; 27.0]	8.9* [5.6; 13.3]	32.2 [26.3; 38.6]
Total	19.4 [16.2; 22.9]	6.5 [4.6; 8.8]	28.8 [25.1; 32.7]

Note. * — differences in comparison with the epidemiological health thresholds (7 %) are statistically insignificant ($p > 0.05$).

Table 2. Herd immunity in healthcare workers

Population groups	Seronegativity to measles virus, % [CI of 95 %]	Seronegativity to rubella virus, % [CI of 95 %]	Seronegativity to mumps virus, % [CI of 95 %]
Healthcare workers (2011)	18.7 [14.5; 23.7]	4.7 [2.6; 7.9]	38.2 [32.6; 44.1]
Healthcare workers (2017)	7.7 [3.6; 14.1]	5.1 [1.9; 10.8]	20.5 [13.6; 29.0]

A considerable proportion of individuals was found to be susceptible to mumps in all studied groups (29.6 % of military recruits, 32.3 % of healthy donors), which is on average twice as high as the epidemic threshold (< 15 %) and may promote infection in the population.

Serum banks accumulate samples obtained before the actual study, making retrospective serological research possible. In our study we used blood serum samples collected from healthcare workers in 2011 when measles incidence rates where increasing in Russia in general and Moscow in particular. Statistical differences ($p < 0.05$) between two sample groups were observed for measles and mumps (Table 2).

Between 2011 and 2017, the proportion of people susceptible to measles considerably decreased and reached the recommended threshold: from 18.7 % in 2001 to 7.7 % in 2017, which may be a result of an additional round of vaccination prompted by the epidemiological situation. The proportion of individuals seronegative to mumps decreased from 38.2 % to 20.5 % but did not reach the recommended threshold, which indicates the need for an additional vaccination round.

DISCUSSION

Based on the obtained results, we conclude that susceptibility to rubella in some population groups and population in general is low and does not go beyond the outbreak threshold (the proportion of seronegative samples in our study was 6.5 %). These results correlate with the official statistics on sporadic rubella incidence of 0.11 per 100,000 population in 2016, with no congenital rubella cases reported [14]. Importantly, over the last 6 years the proportion of seronegative individuals has not changed, which is a good prognostic sign.

There is a risk of measles epidemic, should the pathogen find its way into the country, because in all studied groups the proportion of susceptible individuals (an average of 19.4 %) was above the outbreak threshold. The most vulnerable group was the military recruits (25.5 % of seronegative samples). Another study [15] conducted in contracted soldiers revealed that the proportion of seropositive individuals was 72.4 ± 2.3 %.

The proportion of mumps-susceptible individuals was maximal in healthy donors (32.3 %, with an average rate of 28.8 %). According to the official statistics, mumps rates tend to grow, but only in the case of sporadic incidence [14].

Measles, mumps and rubella are traditionally seen as viral infections with similar epidemiological patterns. The vaccines against these infections are attenuated and administered at the same age (12 months, 6 years). The differences observed by the authors of this works and other researchers [16] regarding the proportion of susceptible individuals may be explained by different immunogenicity of measles, rubella and mumps components contained in the vaccine, whose effectiveness were estimated to be 97 % (67–100 %), 97 % (94–100 %) and 88 % (66–95 %), respectively [17].

CONCLUSIONS

As a method for monitoring herd immunity, serological surveillance allows evaluating vaccination effectiveness, predicting epidemics and planning preventive measures. Our study demonstrates the need for additional immunization campaigns covering the unvaccinated subpopulations, including the armed forces. The large proportion of individuals susceptible to mumps among the studied groups increases the risk of an outbreak in the nearest future. Preventive measures need to be taken now. The situation with rubella is benign, giving hope for complete eradication of this disease in the future.

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THE ROLE OF IMMUNOLOGICAL MEMORY IN ESTABLISHING ANTITUMOR IMMUNITY IN PATIENTS WITH OVARIAN CANCER UNDERGOING NEOADJUVANT THERAPY

Zlatnik EYu[1] ✉, Nerodo GA[2], Ardzha AYu[2], Seliutina ON[1], Bondarenko ES[1]

[1] Laboratory for Immunophenotyping of Tumors,
Rostov Research Institute of Oncology, Rostov-on-Don, Russia

[2] Department of Gynecologic Oncology,
Rostov Research Institute of Oncology, Rostov-on-Don, Russia

Understanding the role of immunological memory mediated by T-lymphocytes in patients with malignant tumors is a pressing issue. This study aimed to assess the contribution of memory T-cells to antitumor immunity in patients with ovarian cancer undergoing neoadjuvant chemoimmunotherapy with recombinant interferon-gamma (rIFNγ). Quantification of central (Tcm) and effector (Tem) memory T-cells (Tm), as well as naive T-lymphocytes (Th0), was done using flow cytometry. Compared to healthy females, untreated cancer patients were found to have more Tm and less Th0 cells in their blood $CD4^+$ and $CD8^+$ T-cell subpopulations. In cancer patients, Tm cells accumulated in the ascitic fluid, exceeding 7.7 times the number of $CD4^+$ Th0 cells and 6.5 times the number of $CD8^+$ Th0 cells, with Tem prevailing over Tcm. After chemotherapy with rIFNγ, blood Th0 decreased in cancer patients, while Tcm dominated the $CD8^+$ Tm subpopulation both in the blood and ascitic fluid. Tem cells were a prevalent cell type in patients who received chemotherapy without interferon-gamma. Decreased Th0 and Tcm prevalence were a positive sign accompanied by a good response to treatment, including lower relapse rates (46.7 % vs. 80 % in controls) and a longer relapse-free period (17.5 ± 1.6 vs. 11.3 ± 1.5 months in controls). Therefore, we conclude that chemoimmunotherapy alters proportions of T-cell subpopulations in the blood and ascitic fluid of patients with ovarian cancer, with Tcm cells prevailing over Tem, which may be one of the mechanisms of rIFNγ (Ingaron) action.

Keywords: ovarian cancer, ascitic fluid, blood, flow cytometry, lympocytes, memory T-cells, Tcm, Tem, Th0, interferon-gamma

✉ **Correspondence should be addressed:** Elena Zlatnik
14-ya liniya, d. 63, Rostov-on-Don, Russia, 344037; elena-zlatnik@mail.ru

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РОЛЬ ИММУНОЛОГИЧЕСКОЙ ПАМЯТИ В ФОРМИРОВАНИИ ПРОТИВООПУХОЛЕВОГО ИММУНИТЕТА У БОЛЬНЫХ РАКОМ ЯИЧНИКОВ НА ЭТАПЕ НЕОАДЪЮВАНТНОГО ЛЕЧЕНИЯ

Е. Ю. Златник[1] ✉, Г. А. Неродо[2], А. Ю. Арджа[2], О. Н. Селютина[1], Е. С. Бондаренко[1]

[1] Лаборатория иммунофенотипирования опухолей,
Ростовский научно-исследовательский онкологический институт, Ростов-на-Дону

[2] Отделение онкогинекологии,
Ростовский научно-исследовательский онкологический институт, Ростов-на-Дону

Изучение роли иммунологической памяти, опосредованной Т-лимфоцитами, при злокачественных опухолях и их лечении — актуальная научная задача. Целью исследования была оценка роли Т-клеток иммунологической памяти в формировании противоопухолевого иммунитета у больных раком яичников на этапе неоадъювантного лечения, включающего химиоиммунотерапию с применением рекомбинантного интерферона-гамма (рИФНγ). Методы: проточная цитометрия для определения центральных (Tcm) и эффекторных (Tem) Т-клеток памяти (Tm) и наивных Т-лимфоцитов (Th0). Результаты: в крови больных по сравнению с донорами обнаружено высокое (Tm) и низкое (Th0) содержание Т-клеток среди $CD4^+$ и $CD8^+$ субпопуляций. В асцитической жидкости происходило накопление Tm, превышающих количество Th0 в 7,7 раза в субпопуляции $CD4^+$ и в 6,5 раза в субпопуляции $CD8^+$, в которой Tem преобладали над Tcm. После химиотерапии с препаратом рИФНγ в крови больных выявлено снижение уровня Th0, в крови и в асцитической жидкости — преобладание Tcm среди $CD8^+$ Tm, а у больных, получавших химиотерапию без иммунотерапии, преобладали Tem. Данные различия были расценены как благоприятные, поскольку сопровождались положительной клинической динамикой: меньшей частотой рецидивирования (46,7 против 80 % в контроле) и более длительным безрецидивным периодом (17,5 ± 1,6 против 11,3 ± 1,5 мес. в контроле). Таким образом, химиоиммунотерапия вызывает перераспределение субпопуляций Т-клеток в крови и в асцитической жидкости при раке яичников в сторону преобладания Tcm над Tem, что может быть одним из механизмов действия препарата «Ингарон» (рИФНγ).

Ключевые слова: рак яичников, асцитическая жидкость, кровь, проточная цитометрия, лимфоциты, Т-клетки памяти, Tcm, Tem, наивные Т-лимфоциты, интерферон-γ

✉ **Для корреспонденции:** Златник Елена Юрьевна
ул. 14-я линия, д. 63, г. Ростов-на-Дону, 344037; elena-zlatnik@mail.ru

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Rapid development of cell technologies promises advancements in antitumour treatment methods. One of those methods is adoptive immunotherapy that implies application of ex vivo activated lymphocytes of peripheral blood and tumor microenvironment lymphocytes (LAK- and TIL-cells), CIK-lymphocytes, DC-vaccination, etc. [1]. In recent years, a number of studies highlighted efficiency of CAR therapy against certain types of tumors [2].

Nevertheless, adoptive immunotherapy in cancer patients often fails to deliver on expectations, which leads to clinicians abandoning the approach both as alternative to chemotherapy or as a complement to it [1]. Such anti-cancer strategies often fail because of immunosuppressive mechanisms active in cancer patients' bodies, the mechanisms that cause imbalance and dysfunction in various subpopulations of lymphocytes, including lymphocytes powering immunological memory. Immunological memory is not a newly discovered phenomenon. It belongs to the adaptive immunity system that includes T- and B-cell links. Memory lymphocytes play an important part in opposing any chronic pathology. As for tumors, of special importance is the immunological memory mediated by T-lymphocytes. In such cases, researchers pay close attention to $CD8^+$ cells, since, compared to their naive counterparts, those memory cells show a much higher incidence of specific cytotoxic T-lymphocytes recognizing tumor-associated antigens [3]. As the example of melanoma melan-A tetramer shows, in cancer patients, naive T-lymphocytes (Th0) and memory T-cells (Tm) respond ex vivo stimulation with tumor antigen differently; $CD45RA^+CCR7^+$ cells do respond and $CD45RO^+CCR7^-$ do not [4, 5]. Some authors believe the level of $CD8^+$ memory cells in a colorectal cancer tumor and other solid tumors is a key factor for survival of patients [6].

Adoption of lymphocytes immunophenotyping methods in the late 20th century lead to discovery of heterogeneity of Tm lymphocytes subpopulation; as is known now, its subdivision depends on the expression of adhesive, chemokine, costimulatory receptors, on the ability to produce cytokines and the response to them, the ability to penetrate through endothelium and other characteristics. These expressions, abilities and characteristics allowed determining basic subpopulations of Tm: central (Tcm) and effector (Tem). Characteristic for the phenotype of these cells is the CD45RO expression and no CD45RA expression, as well as presence of CCR7, CD62L, CD27 and CD28 on Tcm receptors. As for the Tem cells, they have no CCR7, CD62L and CD28 expression and do show that for CD27 [7]. Th0 boast high CCR7, CD62L, CD27, CD28 and CD45RA expression and no CD45RO. Thus, immunophenotyping with a minimal panel of monoclonal antibodies CD62L, CD45RA and CD45RO allows associating T-lymphocyte to a Th0, Tcm or Tem subpopulation.

Chronic antigenic stimulation alters Th0, which results in not just the aforementioned change in their immunophenotype but also in suppression of proliferation and decreased survival rate, as well weaker response to homeostatic cytokines (IL-7, IL-15) and ability to produce IL-2. At the same time, effector functions, cytotoxicity in particular, grow. CD62L and CCR7 receptors expressed by Th0 and Tcm boost their extravasation through high endothelial venules and migration of peripheral lymph nodes to T-dependent zones, while the Tem inhabit peripheral tissues (liver, lungs) and area of inflammation/tumor [8, 9]. These properties make Tem "watchdogs" and Tcm — "guardians" on the systemic level that ensure a quick response to subsequent antigen administration [10, 11].

As for the antitumor activity of effector and central Tm, there are different opinions, but Tcm is a more popular choice [7, 9]. There is evidence proving that, although they act differently, both types of Tm cells make the antitumor protection optimal, i. e. they complement each other [12]. A number of studies states that if there are $CD4^+$ lymphocytes among Tm, they prevent depletion of $CD8^+$ lymphocytes [13]. Thus, it is better to use a heterogeneous mixture of those subsets [12, 13].

In addition to the basic Tm subpopulations, some researchers point to a resident subpopulation (Trm) formed by circulating $CD8^+$ Tm, which expresses additional CD103 receptors in the nidus [14]. Various phenotypic characteristics of $CD8^+$ Tm in different organs have also been reported. For example, peritoneal cavity normally contains only $CD4^+$ and $CD8^+$ effector lymphocytes, but in case of inflammation $CD4^+$ and $CD8^+$ Tm are also found there [14]. Retention of Tm in the microenvironment with their subsequent transformation into Tm-cells can be triggered by appearance of an antigen [15] or the "cytokine explosion" that accompanies inflammation. Earlier, researchers have described Trm cells' activity in the presence of an inflammation [16, 17], but not in tumor growth which might be a different matter.

There is a number of current research papers covering immunological aspects of ovarian cancer treatment; they are part of effort to develop new immunotherapy methods [1, 18]. Such research often leads to some unexpected findings pertaining to immunological memory cells. For example, one study describes a minor subpopulation of Tm expressing B-cell receptor $CD20^+$ together with $IFN\gamma^+$ and $CD8^+$ [19], which allows assuming its tumor growth suppressing qualities.

Normally, Tcm cells dominate in $CD4^+$ subpopulation; the same is true for Tem in $CD8^+$ subpopulation [11]. The numbers of these cells are different in lymphoid and non-lymphoid organs, and their phenotypic and functional characteristics may differ depending on microenvironment [14].

The immune response to a tumor is both system-wide and local, it activates various cell-based and cell-related factors and also their soluble products [18]. However, it is believed that immunocompetent cells and cytokines present in the tumor's microenvironment can both contribute to this tumor's regression and stimulate its growth, especially at advanced stages [20–23].

Treatment of ovarian cancer (OC) remains one of the biggest challenges for oncologists [24]. According to the available data, OC is the fifth most common cancer. Despite the continuous improvement of diagnostic and treatment methods, it still is the deadliest one. Although chemotherapy is quite efficient against OC, the tumor rarely disappears completely and is prone to recur frequently and early. Today, a combination of surgery and chemotherapy is a standard approach to treating OC at III–IV stages. However, currently it is impossible to make full use of cytoreductive operation due to technical difficulties associated with the spread of tumor. In case surgery is not possible, the first stage of treatment is neoadjuvant chemotherapy, and after the surgery comes adjuvant chemotherapy. The most popular combination for a chemotherapy course is Paclitaxel and Carboplatinum with a 3-week interval [25].

Ingaron, recombinant interferon-gamma (rIFNγ) injectable preparation developed by Russian scientists, aims to stimulate cellular immunity and produces antiviral, antiproliferative and immunomodulating effects [26]. Its antitumor activity derives from the ability to activate natural killers (NK cells), cytotoxic T-lymphocytes and macrophages. Given together with cytostatics, Ingaron helps to decrease the resistance of tumor cells to chemotherapy and thus to make the treatment significantly more effective [27]. Research showed Ingaron to be an efficient medication against cervical cancer, breast cancer,

lung cancer, melanoma, colorectal cancer. The preparation worked even when the stages were advanced [28].

This study aimed to assess the contribution of memory T-cells to antitumor immunity in patients with ovarian cancer undergoing neoadjuvant chemoimmunotherapy with recombinant interferon-gamma (rIFNγ)

METHODS

The study was conducted from 2013 to 2017 at the Department of Gynecologic Oncology, and Laboratory for Immunophenotyping of Tumors, Rostov Research Institute of Oncology (RRIO), Rostov-on-Don, Russia. 30 OC patients aged 34 to 77 underwent examination; the mean age was 54.9 ± 1.3 years. All patients had their tumors detected for the first time. The average duration of a case history was 7 months. All patients voluntarily signed informed consent forms, all examinations followed ethical principles set by the Declaration of Helsinki (2013). Ethical committee of RRIO approved the study by the protocol no. 24 of November 23, 2012.

The inclusion criteria were: 18 years old or older; ascitic form, III–IV stage OC verified cytologically or morphologically; no previous special treatment courses.

The exclusion criteria were: expressed comorbidity (previous myocardial infarction, decompensated heart disease, diabetes mellitus); chemotherapy, radiation therapy, surgery, immunotherapy courses underwent before treatment in the context of the study; metastatic lesions in the central nervous system; pregnancy and lactation; any reasons preventing regular treatment and monitoring sessions.

The patients were divided into 2 groups, 15 patients in each. For group 1 (treatment group) chemotherapy was combined with rIFNγ immunotherapy (ChIT), group 2 (control group) got chemotherapy (ChT) only.

Comparative analysis of the groups revealed that both had 11 (73.3 %) stage III OC patients and 4 (26.7 %) stage IV OC patients. The differences were statistically insignificant ($p > 0.05$). The majority of patients were aged 50–59 years: 5 (33.3 %) in the treatment, 6 (40.0 %) in the control group. No statistically significant difference detected. ECOG-WHO scale was applied to assess the general condition of patients; most scored 2 points (8 (53.3 %) and 9 (60.0 %) patients in treatment group and control group, respectively). The differences were not statistically significant. Thus, age, OC stages and general condition were identical for both groups, which allows making the comparative analysis.

The comparison group consisted of 20 practically healthy women of the same age who had blood samples taken.

The patients were examined as their treatment progressed, before and 3 weeks after ChT (15 patients) and ChIT (15 patients). The 3-week interval matched that between treatment courses. The patients received two to three courses of polychemotherapy: Carboplatinum (AUC-6), intravenously (dropping), Paclitaxel 175 mg/m^2 intravenously (dropping), 21 days between the courses. ChIT regimen was as follows: rIFNγ (Ingaron by Pharmaclone, Russia) injected intramuscularly, 500,000 IU on day 1, 1,000,000 IU on days 2, 3 and 5, and day 4 was for Paclitaxel + Carboplatinum (polychemotherapy). Age, OC stages and general conditions were identical for both groups.

Further, all patients underwent surgery depending on the results of the treatment. It could be total hysterectomy with salpingo-oophorectomy, omentum resection or extirpation, salpingo-oophorectomy. Post-operation, treatment group patients received adjuvant ChIT: Ingaron, intramuscularly, same regimen started on the 9th day. The patients had 2 Ingaron cycles and then 4 chemotherapy cycles, which made the entire adjuvant treatment course included 6 cycles. Control group patients received 6 ChT cycles.

Complete or partial regression and the general effect (tumor regression and stabilization) and progression values as defined by WHO were criteria for assessment of the effect produced by neoadjuvant therapy.

The post-treatment follow-up period was 3 years, with tumor recurrence time registered.

Blood and ascitic fluid (AF) samples were taken before the treatment and at its stages. The total number of blood samples examined was 98, number of AF samples equaled 47.

Blood lymphocytes and AF flow cytofluorimetry. The device used for the purpose was BD FACSCanto II flow cytometer (Becton Dickinson, USA). It features two lasers emitting at 488 nm and 633 nm (fluorophores excitation wavelengths) and allows using up to 6 monoclonal antibodies in one tube simultaneously. Monoclonal antibodies were conjugated with the following fluorochromes: FITC, PE, PerCP-Cy5.5, PE-Cy7, APC, APC-Cy7. The cytometer was set up with the help of standard BD FACS 7-color setup beads (BD Biosciences, USA).

Sample preparation and immunophenotypic staining. To assess expression of receptors, researchers resorted to immunophenotypic staining. It was done with fluorescently-labeled antibodies as prescribed by the protocol supplied by the manufacturer. For each examination, 2 tubes with the following sets of monoclonal antibodies BD Multitest (Becton Dickinson, USA) were used:

1. CD45RA FITC/CD45RO PE/CD3 PerCP/CD4 APC (cat. #340571) or CD45RA FITC/CD45RO PE/CD3 PerCP/CD8 APC (cat. #340574);
2. CD45RA FITC/CD62L PE/CD3 PerCP/CD4 APC (cat. #340977).

This panel of antibodies allows to count the content of Th0, Tcm and Tem ($CD62L^+CD45RA^+CD45RO^-$, $CD62L^+CD45RA^-CD45RO^+$ and $CD62L^-CD45RA^-CD45RO^+$ respectively) among $CD4^+$ and $CD8^+$ Tm subpopulations. At least 50,000 cells were accumulated in each sample for data analysis.

Gating tactics and data analysis. The lymphocyte region was determined with the help of Dot Plot chart by direct (relative cell size) and lateral (cell structure) light scattering parameters. The share of T-helpers and cytotoxic T-lymphocytes in the overall lymphocytes population was calculated within this range by markers CD3, CD4 and CD8. Next, we analyzed the share of T-lymphocytes with phenotypes $CD4^+CD45RO^+CD45RA^-$, $CD8^+CD45RO^+CD45RA^-$, $CD4^+CD45RA^+CD62L^+$, $CD8^+CD45RA^+CD62L^+$ in the populations of T-helpers and cytotoxic T-lymphocytes. For each sample, Tm/Th0 coefficients were calculated for $CD4^+$ and $CD8^+$ subpopulations. Tcm and Tem levels were taken as a percentage of the number of Tm belonging to $CD4^+$ and $CD8^+$ subpopulations, then the Tem/Tcm coefficient was calculated. Fig. 1 and 2 show examples of blood and AF flow cytofluorimetry results.

Mathematical and statistical processing methods. BD FACSDiva Software (Becton Dickinson, USA) did mathematical processing of the data. Statistica 8.0 for Windows and MS Excel were the tools that enabled the results analysis and statistical processing. The threshold of statistical significance of differences was $p < 0.05$. The Wilcoxon test was also used; the χ^2 fitting criterion helped to assess reliability of differences between frequency of development of total and overall effect and frequency of recurrence.

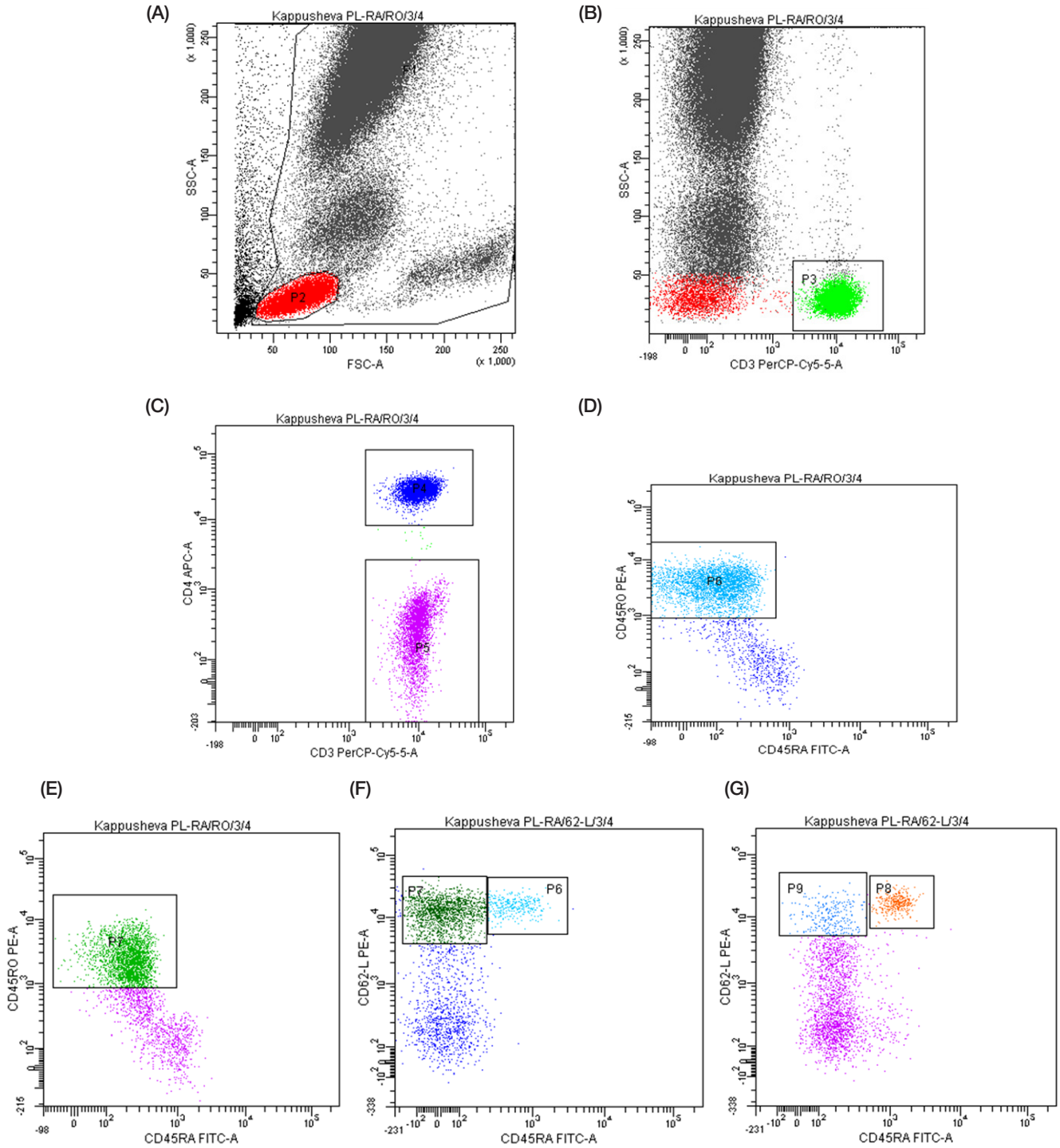

Fig. 1. Peripheral blood Tm, flow cytometry: **(A)** — lymphocyte region among blood cells; **(B)** — T-lymphocytes population; **(C)** — distribution of CD4 and CD8; **(D)** — distribution of CD45RA and CD45RO after logical restriction by CD4; **(E)** — distribution of CD45RA and CD45RO after logical restriction by CD8; **(F)** — distribution of CD45RA and CD62L after logical restriction by CD4; **(G)** — distribution of CD45RA and CD62L after logical restriction by CD8

RESULTS

Tables 1–4 and in Fig. 3 and 4 show the results of the study. We found some differences in content of Tm and Th0 lymphocytes in biological fluids samples. As seen in Table 1, blood of OC patients has a higher level of Tm among $CD3^+CD4^+$ cells and a lower level of Th0 among $CD3^+CD8^+$ cells compared to those seen in healthy women; the difference is statistically significant ($p < 0.05$). Comparison of levels of Tm in blood and AF of OC patients revealed that these cells are much more common in AF ($p < 0.05$). In contrast, the levels of Th0 in AF were statistically significantly lower when compared to those seen in the patients's blood ($p < 0.05$) (Table 1).

There were no statistically significant differences between $CD4^+$ and $CD8^+$ cell levels in blood and AF, although levels of lymphocytes and monocytes in AF significantly exceeded those seen in blood. Despite the significantly higher level of lymphocytes in AF (52.4 ± 5.5 compared to 17.4 ± 2.7 % in patients' blood), the contents of their main subpopulations in these biological fluids were not statistically different: the level of $CD4^+$ cells in blood was 49.2 ± 3.1 %, that in the AF — 48.7 ± 3.2 %; $CD8^+$ levels were 18.4 ± 2.7 and 22.6 ± 3.3 %,

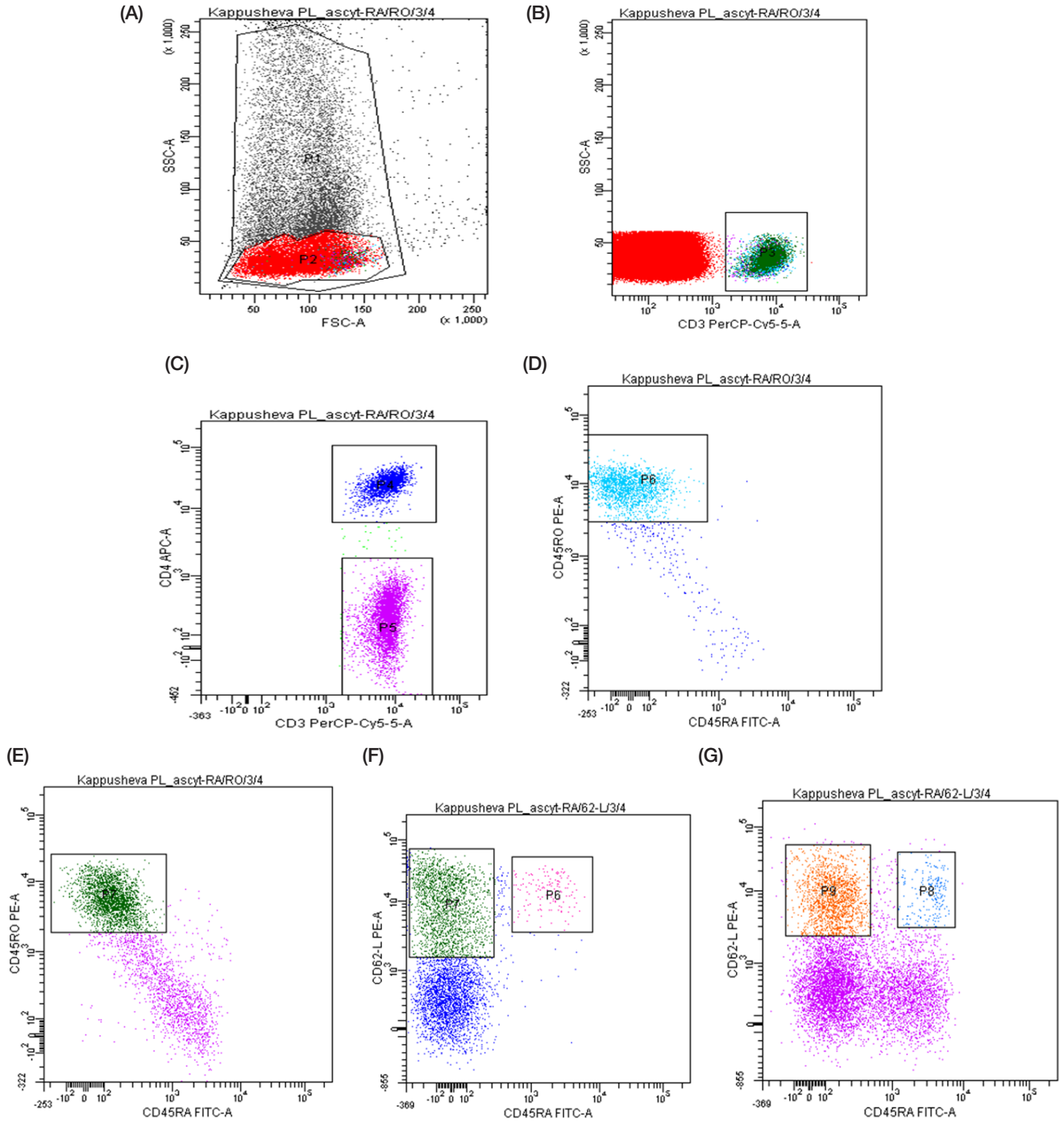

Fig. 2. AF Tm, flow cytometry: **(A)** — lymphocytes region; **(B)** — T-lymphocytes population; **(C)** — distribution of CD4 and CD8; **(D)** — distribution of CD45RA and CD45RO after logical restriction by CD4; **(E)** — distribution of CD45RA and CD45RO after logical restriction by CD8; **(F)** — the distribution of CD45RA and CD62L after logical restriction by CD4; **(G)** — distribution of CD45RA and CD62L after logical restriction by CD8

respectively. The $CD4^+/CD8^+$ index for AF was also on par with that for blood of patients, i. e. there were no statistically significant differences there. However, the ratio of Tm/Th0 found in both T-cell subpopulations was significantly higher in AF of patients than in their blood (7.7 ± 1.0 vs. 2.9 ± 0.5 for $CD4^+$ cells and 6.5 ± 1.3 vs. 2.1 ± 0.3 for $CD8^+$ cells; in both cases, $p < 0.05$). This means there are markedly more Tm than Th0 in the tumor nidus than in the peripheral blood.

We also found some differences in content of Tm subpopulations in the examined biological fluids (Table 2).

Table 2 shows that Tem levels among subpopulations of $CD4^+$ and $CD8^+$ cells in AF are statistically significantly higher than those in blood of patients and in blood of healthy women ($p < 0.05$). As for the content of Tcm, the differences between AF of patients and blood of healthy women were found for $CD8^+$ only. It is interesting that there were no statistically significant differences in the studied subpopulations in blood of patients and healthy individuals.

Tem levels in $CD4^+$ and $CD8^+$ subpopulations were similar in most cases, whereas the share of Tm in $CD4^+$ subpopulation was 2–2.3 times higher than in $CD8^+$ subpopulation. In blood and AF samples, Tem dominated over Tcm in $CD8^+$ cell subpopulation (over 1.0), and Tcm dominated over Tem in $CD4^+$ subpopulation (below 1.0) (Fig. 3).

Thus, comparison of systemic and local cellular immunity indices seen in AF of OC patients showed that Tm dominate in

patients' blood when put against that of healthy women and in the patients' AF when compared to their peripheral blood. Most of the $CD4^+$ Tm were Tcm while the majority of $CD8^+$ Tm were Tem, which accumulated in patients' AF.

Neoadjuvant treatment lead to a complete regression of tumor in 4 patients (26.7 %) of the treatment group (ChT plus rIFNγ) and in 1 (6.7 %) belonging to the control group. Assessment of the overall effect of treatment revealed statistically significant differences in data describing treatment group and control group (87.0 and 30.0 %, respectively). Only 1 patient of the control group had the tumor progressing (6.7 %).

After 2–3 cycles of neoadjuvant polychemotherapy, 12 (80.0 %) patients of the treatment group underwent full-scale surgery (total hysterectomy with salpingo-oophorectomy, omentum resection or extirpation). In control group, only 6 patients were in a state allowing this degree of intervention. The difference is significant ($p < 0.05$). 3 patients (20.0 %) of the treatment group had incomplete surgery that included oothecoma removal and omentum resection; in the control group, the same operations were performed on 8 patients (53.3 %), and 1 patient had a trial laparotomy.

The post-adjuvant treatment follow-up period was 3 years. Recurrence in the treatment group was 46.7 % while that in the control group was 80.0 % (7 and 12 patients, respectively). The average time of the recurrence was 17.5 ± 1.6 months and 11.3 ± 1.5 months, respectively. The differences are significant ($p < 0.05$).

Only the patients that had the same kind of surgery (full-scale surgery) were picked for immunological examination.

Dynamics of levels of Tm and Th0 in blood of patients from both groups can be seen in Table 3. There are some differences there. Worth a special note is the decrease of levels of naive Th0 in treatment group patients (ChT plus rIFNγ), which became statistically significant by the end of the treatment ($p < 0.05$). In the control group, the level of these cells did not differ from the initial one during the entire follow-up period.

The Tm/Th0 ratio was growing (and reached statistical significance) in the treatment group and not in the control group (Fig. 4). Thus, the $CD8^+$ cells Tm/Th0 ratio increased from 2.5 ± 0.5 to 3.4 ± 0.7 in patients of the treatment group, while in the control group it went down from 1.7 ± 0.3 to 1.4 ± 0.3. The difference is significant ($p < 0.05$). As for the $CD4^+$ subpopulation, the growth was 2.5-fold in the treatment group and in the control group it did not change.

During treatment, Tm content was not significantly different in treatment and control groups (Table 3), but assessment of dynamics revealed such differences in $CD8^+$ Tm subpopulation (Table 4). In the control group, the level of Tcm gradually decreased and by the end of the treatment the difference acquired statistical significance ($p < 0.05$). Tem levels did not change.

ChIT that included rIFNγ initially led to similar changes, but as the treatment approached completion, the volume of Tcm was greater and Tem lower in blood of treatment group patients that those in blood of control group patients (Table 4). In other words, Tcm dominated in blood of the treatment group patients and Tem were the majority in the control group patients' blood. The Tem / Tcm ratio was 2.27 ± 0.4 and 0.62 ± 0.18, respectively, $p < 0.05$.

The dynamics of levels of the studied subpopulations in AF could only be registered when the fluid accumulated, i. e. during the preoperative period. It was found that the Tm/Th0 levels ratio in $CD4^+$ lymphocytes subpopulation increased from 7.7 ± 1.0 to 13.0 ± 1.5 ($p < 0.05$); the data describes treatment group, the increase is statistically significant. AF of patients of the same group has also shown changes in Tm $CD8^+$ subpopulation: the level of Tem decreased (13.6 ± 4.6 compared to 42.1 ± 4.1 % in the control group, $p < 0.05$), and the volume of Tcm tended to increase. The total number of lymphocytes in AF after ChIT was 42.2 ± 7.8, while after ChT it was 31.1 ± 7.1 % (the differences are statistically insignificant); However, ChT lead to the reduction in their numbers compared to the initial counts, the difference here being statistically significant (52.4 ± 5.5 %, $p < 0.05$). Other statistically significant differences include those describing $CD3^+$ and $CD4^+$ cell levels: ChIT brought them up to 82.1 ± 6.3 and 57.6 ± 5.7, respectively, while ChT produced a more modest growth of 65.4 ± 6.5 % and 42.6 ± 2.5 %, respectively; in both cases $p < 0.05$.

Thus, the content of Tm in both $CD4^+$ and $CD8^+$ subpopulations in patients' AF was greater than that in their blood, while Th0 was smaller; among $TmCD8^+$, Tem were prevalent. The study revealed that adding Ingaron (rIFNγ) to a chemotherapy course, thus making it a chemoimmunotherapy

Table 1. Tm and Th0 subpopulations of $CD4^+$ and $CD8^+$ in blood and AF of OC patients

Samples	Tm, %		Th0, %	
	$CD4^+$	$CD8^+$	$CD4^+$	$CD8^+$
Blood of healthy women (n = 20)	55.0 ± 3.7	35.0 ± 4.3	23.4 ± 3.2	27.7 ± 3.7
Blood of OC patients (n = 30)	64.5 ± 2.3*	40.8 ± 5.1	22.4 ± 4.1	18.5 ± 2.7*
AF of OC patients (n = 30)	79.2 ± 4.0*,**	57.5 ± 3.1*,**	10.2 ± 1.6*,**	9.5 ± 2.5*,**

Note. * — significant differences from blood of healthy donors ($p < 0.05$); ** — significant differences from blood of patients ($p < 0.05$). Hereinafter, in tables 2–4 reliability of the differences was calculated through the Wilcoxon test.

Table 2. Central and effector Tm with $CD4^+$ and $CD8^+$ phenotype in blood and AF of OC patients

Samples	Tm $CD4^+$		Tm $CD8^+$	
	Tcm, %	Tem, %	Tcm, %	Tem, %
Blood of healthy women (n = 20)	41.7 ± 2.5	17.8 ± 2.3*	17.7 ± 2.5*	24.1 ± 3.6*
Blood of OC patients (n = 30)	42.0 ± 3.1	24.8 ± 1.8*	22.0 ± 1.5	28.2 ± 3.2*
AF of OC patients (n = 30)	47.1 ± 3.3	39.8 ± 3.9	27.0 ± 2.0	42.1 ± 4.1

Note. * — significant differences from AF ($p < 0.05$).

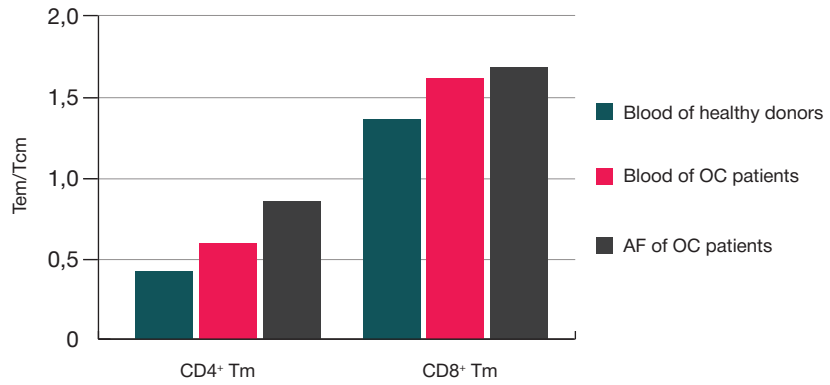

Fig. 3. Tem / Tcm ratio in CD4+ and CD8+ subpopulations of memory T-cells

course, maintains the opposite dynamics of Tem and Tcm cells in patients' blood and AF.

DISCUSSION

The biological role Tm play in resisting cancer is important. The volumes and ratios of these cells can be changed through immunotherapy. Within the context of this study, we monitored and registered the levels of Tm (including Tcm, Tem) and Th0 in blood and ascitic fluid (AF) of OC patients before treatment and during chemoimmunotherapy. Earlier, we registered and described a higher content of lymphocytes (with Tm dominating) in AF of patients [29]; this study goes further and discovers the prevalence of Tem there, mostly belonging to CD8+ subpopulation. Although it is not yet possible to establish their specificity and substantiate the hypothesis of their antitumor activity, the published research data available indicate that, since the expansion of lymphocytes as part of a lymphoid infiltrate in the tumor (tumor-infiltrating lymphocytes) is clonal, antigen-specific lymphocytes should dominate in the tumor microenvironment [6]. Judging by the noticeable spread of tumor, OC patients do not develop effective immune reactions, which may be caused by some peculiar features of the tumor microenvironment. It appears that tumor cells in AF activate peritoneal macrophages and monocytes, which leads to hyperproduction of cytokines possessing pro-tumor properties [21]. AF monocytes are a potential source of type 2 macrophages that support tumor growth by autocrine production of VEGF, EGF, TGFß, IL-6, IL-8, IL-10, supported by hypoxia developing in tumor tissue [23, 30]. These same cytokines, as well as chemokines that can be produced by tumor cells, stimulate the migration of lymphocytes into the peritoneal cavity. According to the published data, there are natural T-regulatory cells (T-regs) among them, and they suppress immunity and boost tumor growth. OC patients have more such T-regs than healthy women [31], and the cells are much more abundant in patients' AF than in their blood [32]. They probably block the activity of T-lymphocytes, Tm in particular and other lymphocytes (NK-, CD8+) in general. Those lymphocytes, although abundant, seem to be hindered in their functions, as shown by the example of CD8+ Tm chronically stimulated in the presence of viral infections [7, 9, 33, 34]. Unlike T-regs, Tm die on schedule (apoptosis) when interacting with FasL expressed by endotheliocytes of tumor vessels; this may be one of the self-protective mechanisms the tumor has [35]. Perhaps, some measures countering local immunosuppression could allow effective inclusion of cellular factors into antitumor immune response that would rise the effectiveness of OC treatment.

As we have reported earlier, Ingaron added to a chemotherapy course causes a decrease in the level of Th0 in OC patients and otherwise positively affects their immunity [36]. In this study, we focused on learning more about Tm in OC patients. We have found that introduction of rIFNγ ensures domination of Tm over Th0 in blood and AF of OC patients after chemoimmunotherapy. It also provokes redistribution of Tm CD8+ and brings around more Tcm, which is not seen in patients that receive chemotherapy without rIFNγ. In part, this may be the reason behind the improved clinical effect observed when rIFNγ joins chemotherapy. Although the reports published to date indicate that repeated administration of the antigen provokes proliferation and increases cytotoxicity both of Tcm and Tem (part of CD8+ T-cell subpopulation), it is believed that Tcm has these properties more pronounced and also shows a more active interaction with antigen-presenting cells, higher antigen-induced proliferation, generation of cytotoxic T-effectors and homing into secondary lymphoid organs [7, 37]. This suggests the advantage tumor-specific CD8+ Tcm have when compared to Tem as antitumor factors. Thus, the data we have received can be interpreted as one of the therapeutic mechanisms Ingaron offers against ascitic forms of OC.

Table 3. Dynamics of Tm and Th0 levels, blood of OC patients, treatment and control groups

Group of patients	Treatment term	Tm, %		Th0, %	
		CD4+	CD8+	CD4+	CD8+
Treatment (n = 12)	before treatment	65.8 ± 4.2	40.9 ± 5.2	20.5 ± 3.5*	17.2 ± 1.6*
	after 2–3 ChIT cycles	64.6 ± 6.1	37.7 ± 4.5	15.7 ± 3.4	16.5 ± 2.3
	after full ChIT course	72.8 ± 5.9	35.7 ± 5.1	9.7 ± 2.7	10.4 ± 2.6
control (n = 6)	before treatment	66.3 ± 4.4	38.1 ± 4.7	23.6 ± 4.6	23.6 ± 5.6
	after 2–3 ChT cycles	59.1 ± 7.1	31.6 ± 4.5	16.8 ± 3.9	18.8 ± 3.2
	after full ChT course	63.2 ± 7.3	37.5 ± 7.2	24.4 ± 6.2*	27.3 ± 7.5*

Note. * — significant differences from the parameters of the treatment group after full ChIT course ($p < 0.05$).

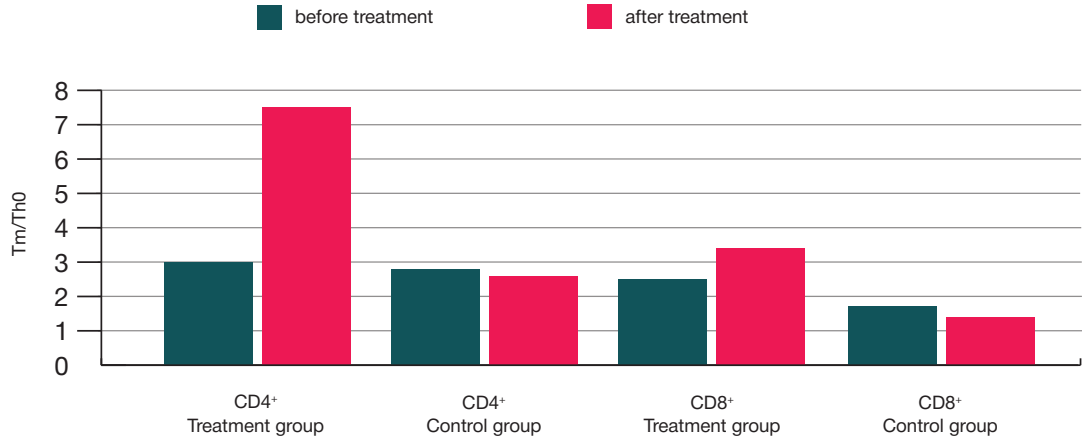

Fig. 4. Changes in Tm / Th0 ratio, CD4+ and CD8+ subpopulations, blood of OC patients before and after treatment

Table 4. Dynamics of Tcm and Tem levels, blood of OC patients, treatment and control groups

Group of patients	Treatment term	Tm CD4+		Tm CD8+	
		Tcm, %	Tem, %	Tcm, %	Tem, %
Treatment (n = 12)	before treatment	43.0 ± 2.5	26.3 ± 3.8	24.4 ± 3.6	25.8 ± 3.3
	after 2–3 ChIT cycles	39.8 ± 4.1	26.8 ± 3.3	13.3 ± 3.4*	22.6 ± 3.7
	after full ChIT course	42.8 ± 3.8	28.4 ± 5.1	22.2 ± 2.9**	13.9 ± 3.6*,**
Control (n = 6)	before treatment	42.3 ± 3.6	25.5 ± 2.8	22.5 ± 2.3	30.6 ± 4.9
	after 2–3 ChT cycles	45.3 ± 4.7	23.7 ± 3.7	17.8 ± 4.0	25.3 ± 2.9
	after full ChT course	43.1 ± 6.1	23.8 ± 4.7	12.6 ± 2.2*	28.7 ± 3.3

Note. * — significant differences from values seen before treatment ($p < 0.05$); ** — significant differences from the corresponding values of the control group ($p < 0.05$).

CONCLUSIONS

Before treatment, OC patients (ascitic forms) have Tcm dominating in CD4+ subpopulation of Tm and Tem dominating in CD8+ subpopulation of Tm, which accumulate in ascitic fluid (AF). Despite the high levels of Tm in blood and AF, their function seems to be injured and therefore it is important to study the ways to correct it.

Compared to patients that received plain chemotherapy, those who had chemoimmunotherapy course with recombinant interferon-gamma showed better clinical effects: decreased volume of Th0 in blood, domination of Tcm over Tem in CD8+ Tm subpopulation. Since the like changes take place in AF too, such modulation of the tumor's immunological microenvironment can boost the clinical effect and, in particular, ensure longer recurrence-free period in OC patients.

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LONG-TERM EFFECT OF HIGH CYCLOPHOSPHAMIDE DOSES ON THE REPERTOIRE OF T-CELL RECEPTORS OF PERIPHERAL BLOOD T-LYMPHOCYTES IN PATIENTS WITH AUTOIMMUNE VASCULITIS

Merzlyak EM[1], Kasatskaya SA[1], Sosnovskaya AV[2], Israelson MA[1], Staroverov DB[1], Nakonechnaya TO[1], Novikov PI[2], Chudakov DM[1], Britanova OV[1] ✉

[1] Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry of the Russian Academy of Sciences, Moscow, Russia

[2] I. M. Sechenov First Moscow State Medical University, Moscow, Russia

Although mechanisms underlying development of autoimmune vasculitis and polyangiitis remain understudied, these pathologies are already known to be largely mediated by T-lymphocytes. Cyclophosphamide (CF) is widely used to treat autoimmune diseases. Lymphoid cells in general (T, B, and NK cells) and naive T-lymphocytes in particular are highly sensitive to CF. In this work we analyzed the repertoires of T-cell receptors (TCRs) in the peripheral blood of young (aged 24 to 35 years, n = 4) and elderly (aged 52 to 68 years, n = 5) patients with ANCA-associated vasculitis (Wegener granulomatosis and Churg–Strauss syndrome) treated with high doses of CF > 3 years before the study. The control group included 7 young and 14 elderly healthy individuals. We revealed no TCR variants previously reported as typically found in patients with ANCA-associated vasculitis. Relative frequency of “public" (often found in a population, largely formed during an embryonic period) TCR variants in the repertories of young patients was significantly lower than in the repertories of healthy donors of the same age, and was similar to the elderly healthy donors. We hypothesize that CF-treatment eliminates substantial proportion of naïve T-cells in the young donors, that contains "public" TCR variants of fetal origin. Long-term consequences of such changes in the structure of T-cell immunity require further investigations.

Keywords: autoimmune disease, autoimmune vasculitis, cyclophosphamide, T-cell receptor, naive T-lymphocyte, high-throughput sequencing

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✉ **Correspondence should be addressed:** Olga Britanova
ul. Miklukho-Maklaya, d. 16/10, Moscow, Russia, 117997; olbritan@gmail.com

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ИССЛЕДОВАНИЕ ДОЛГОСРОЧНОГО ЭФФЕКТА ВЫСОКИХ ДОЗ ЦИКЛОФОСФАМИДА НА РЕПЕРТУАР Т-КЛЕТОЧНЫХ РЕЦЕПТОРОВ Т-ЛИМФОЦИТОВ ПЕРИФЕРИЧЕСКОЙ КРОВИ У ПАЦИЕНТОВ С АУТОИММУННЫМИ ВАСКУЛИТАМИ

Е. М. Мерзляк[1], С. А. Касацкая[1], А. В Сосновская[2], М. А. Израельсон[1], Д. Б. Староверов[1], Т. О. Наконечная[1], П. И. Новиков[2], Д. М. Чудаков[1], О. В. Британова[1] ✉

[1] Институт биоорганической химии имени академиков М. М. Шемякина и Ю. А. Овчинникова РАН, Москва

[2] Первый Московский государственный медицинский университет имени И. М. Сеченова (Сеченовский Университет), Москва

Механизмы развития аутоиммунных васкулитов и полиангиитов мало изучены, однако известно, что патогенез этих заболеваний в значительной степени опосредуется Т-лимфоцитами. Циклофосфамид (ЦФ) широко используется для лечения аутоиммунных заболеваний. Клетки лимфоидного ряда (Т, В и NK-клетки), и в особенности наивные Т-лимфоциты, обладают высокой чувствительностью к ЦФ. Мы проанализировали репертуары Т-клеточных рецепторов (T-cell receptors, TCRs) периферической крови молодых (24–35 лет, n = 4) и пожилых (52–68 лет, n = 5) пациентов с синдромами Вегенера и Чарга–Стросса, получавших не ранее чем за 3 года до начала исследования ЦФ в высоких дозах. В контрольную группу включили здоровых доноров: 7 молодых и 14 пожилых людей. Мы не выявили описанных ранее вариантов TCRs, характерных для ANCA-ассоциированных васкулитов. Представленность «публичных» (часто встречающихся в популяции, в значительной степени формирующихся в эмбриональном периоде) вариантов TCRs в репертуарах молодых пациентов оказалась существенно ниже, чем в репертуарах здоровых доноров того же возраста, и была близка к таковой пожилых здоровых доноров. Мы предполагаем, что терапия высокими дозами ЦФ элиминирует значительную часть наивных Т-лимфоцитов молодых доноров, содержащих публичные варианты TCR эмбрионального происхождения. Отдаленные последствия таких изменений в структуре Т-клеточного иммунитета требуют дальнейшего изучения.

Ключевые слова: аутоиммунные заболевания, аутоимунный васкулит, циклофосфамид, Т-клеточный рецептор, наивный Т-лимфоцит, высокопроизводительное секвенирование

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✉ **Для корреспонденции:** Британова Ольга Владимировна
ул. Миклухо-Маклая, д. 16/10, г. Москва, 117997; olbritan@gmail.com

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Immunosupressants have become a common treatment option for autoimmune diseases. One of such drugs, the cytotoxic cyclophosphamide (CPH), whose immunosuppressive properties are still understudied, is used for treating severe pathologies, including autoimmune vasculitis. CPH is metabolized in the liver into 4-hydroxycyclophosphamide that readily diffuses into the cells and then converts into either phosphoramide mustard, an active cytotoxic compound, or inert carboxyphosphamide (given that the cell is rich in aldehyde dehydrogenase). Lymphoid cells, including T- and B-lymphocytes and natural killer cells, are low in aldehyde dehydrogenase and die when exposed to high doses of cyclophosphamide. But primitive hematopoietic cells are rich in this enzyme and therefore resistant to CPH. Thus, CPH has a pronounced immunosuppressant effect, but is not myeloablative: stem cells survive exposure to CPH, retaining their hematopoietic activity, which renders bone marrow transplantation irrelevant [1]. High doses of CPH have been clinically confirmed to induce remission in patients with different types of autoimmune disorders (acute aplastic anemia, myasthenia gravis, systemic scleroderma, etc.), but 5-year relapse-free rates following the treatment with CPH are as low as 10 % [2].

There are reports about the specific effect of CPH on naive T-cells. For example, Gladstone et al. [3] have demonstrated a significant reduction in naive $CD45RA^+CD4^+$ T-lymphocytes in comparison with $CD45RO^+CD4^+$ memory cells in patients with multiple sclerosis who received high doses of CPH. Almost all the participants retained their immune status, including the resistance to the infections they had had in childhood, which is mediated by memory cells. Gladstone's findings indicate that memory cells are less sensitive to CPH than naive T-lymphocytes.

The evolution of high-throughput sequencing has enabled deep profiling of T-cell receptors (TCR) repertoire. Combined with an immunofluorescence staining, this technology has yielded a few important discoveries about age-driven changes in the adaptive immunity. In our previous work we have analyzed a wide range of samples, including umbilical cord blood and peripheral blood of centenarians, to reveal a correlation between the reduction in naive T-lymphocytes and the decreasing diversity of the TCR repertoire. We have also described some changes in the structure of the TCR repertoire occurring throughout the life [4].

Mechanisms underlying the development of vasculitis remain understudied. The primary diagnostic marker for this disease is anti-neutrophil cytoplasmic antibodies (ANCAs). Patients with Wegener granulomatosis (granulomatosis with polyangiitis, GPA) have antibodies to proteinase-3 (PR-3), while patients with Churg–Strauss syndrome (eosinophilic granulomatosis with polyangiitis, EGPA) and microscopic polyangiitis have antibodies to myeloperoxidase. ANCAs are thought to be implicated in the pathogenesis of vasculitis through the interaction with the antigen and stimulation of neutrophil degranulation, which causes endothelial and therefore vascular damage [5]. T cells also actively contribute to the development of the disease. The afflicted patients have increased counts of activated $CD4^+$ and $CD8^+$ T-lymphocytes circulating in the peripheral blood and elevated levels of proinflammatory factors implicated in their activation. Activated T-cells are also known to participate in granuloma formation [6, 7]. It has been shown that glomerular crescent formation is suppressed in animals with depleted T-lymphocytes [8].

A question remains about the effect of CPH on different functional subsets of blood cells and its long-term impact on the adaptive immunity. In the recent years a number of works have been published concerning the role of CPH in TCR reconstitution after allogeneic blood or marrow transplantation. It has been shown that high doses of CPH, administered on days 3 and 4 after the intervention, significantly reduce the risk of both acute and chronic "graft-versus-host" disease and viral infections, including Epstein-Bar-related lymphoproliferative posttransplantation conditions [9]. Kanakry et al. demonstrate that the first to recover after CPH treatment are effector memory cells whose diversity is, however, impoverished in comparison with that of healthy donors' cells [10].

In this work we employed high-throughput sequencing to study repertoires of peripheral blood TCR obtained from 9 patients with vasculitis previously treated with high doses of cyclophosphamide. Specifically, we compared TCR beta-chains repertoires in these patients and healthy controls and attempted a search for disease-associated variants of TCR and expression patterns of well-represented V- and J-beta TCR segments. We also evaluated the long-term effect of CPH on the repertoire of peripheral blood TCR.

METHODS

Selecting the participants

Two groups were formed: a group of patients diagnosed with vasculitis who had undergone treatment with high doses of cyclophosphomide > 3 years before the study and a group of healthy volunteers (controls). Each group was divided into subgroups of young (24–35 years) and elderly (52–68 years) individuals. The subgroup of young patients consisted of 3 males and 1 female, the subgroup of elderly patients included 4 males and 3 females; the control group consisted of 6 males and 8 females.

The study was approved by the Ethics Committee of Dmitry Rogachev National Research Center of Pediatric Hematology, Oncology and Immunology, Moscow (Protocol No. 2013-5/4). Informed consent was obtained from all participants.

Isolation of mononuclear cells and immunofluorescence staining

Eight milliliters of blood were collected from each participant for blood count and immunofluorescence staining performed as described in [11]. A hundred microliters of peripheral blood were incubated with the following monoclonal antibodies: FITC anti-human CD45RA (eBioscience, Thermo Fisher Scientific, USA), CD27-PC5 (eBioscience), CD4-PE (Beckman Coulter, USA), and CD8-eFluor 405 (eBioscience), and then lysed using Optilyse-C (Beckman Coulter). The samples were analyzed in the flow cytometer Cytomics CF500 (Beckman Coulter). Peripheral blood mononuclear cells were isolated from 6 ml of blood by Ficoll-Paque density gradient centrifugation (PanEco, Russia).

Isolation of total RNA, preparation of TCR cDNA libraries and sequencing

Total RNA was isolated using Trizol Reagent (Invitrogen, USA) according to the manufacturer's protocol. RNA yield was measured in the QuBit 3 fluorometer (Invitrogen) by intercalator fluorescence intensity. Reaction quality was evaluated by gel electrophoresis.

TCR cDNA libraries were obtained as described in [11]. TCR beta chain cDNA was synthesized using the Mint kit

(Evrogen, Russia) according to the manufacturer's protocol and 1.5 μg of RNA per reaction. Depending on the amount of the isolated RNA, 4 to 6 synthesis reactions were run per sample. The primers used for cDNA synthesis, the 5′-template switch adaptor carrying 12 random nucleotides and the amplification protocol are described in [11]. Owing to the use of the 5′-adaptor, each synthesized cDNA received a unique barcode. The schematic of the experiment is shown in Fig. 1.

The obtained cDNA was amplified, purified using the Cleanup Standard kit (Evrogen) and concentrated. Concentrations of the obtained libraries were measured in QuBit 3 (Invitrogen). cDNA molecules were ligated to Illumina TruSeq adaptors (Illumina, USA). Libraries were sequenced using Illumina HiSeq 2000 (Illumina) to generate 2 x 150 nt paired-end reads.

Bioinformatic analysis of sequencing data

Demultiplexing of sequencing data and assembly of reads grouped by their molecular identifier (barcode) were done using the MiGEC pipeline [12] as described in [11]. CDR3 extraction and assembly of T-cell clonotypes were aided by the MiXCR software [13]. Qualitative analysis of clonotypes (V- and J-usage, repertoire overlap) and diversity estimates were conducted using VDJtools [14].

Data were statistically processed in the GraphPad Prism5 (Graph Pad Software, USA). Public clonotypes were analyzed using R algorithms.

RESULTS

Flow cytometry of peripheral blood T-cell subsets

Flow cytometry was used to quantify peripheral blood naive $CD3^+$ cells (phenotype $CD3^+CD45RA^+CD27^+$) and naive $CD4^+$/$CD8^+$ lymphocytes in the samples of patients with vasculitis treated with high doses of CPH. The results were compared to previously obtained data on healthy donors of different ages [4, 11]. No significant difference was observed in the proportion of naive T-lymphocytes between diseased and healthy individuals of the same age (Fig. 2, A).

Analysis of TCR diversity by high-throughput sequencing

Molecular barcoding of cDNA libraries allowed us to trace and correct substitution errors occurred during amplification and sequencing. To prepare cDNA libraries necessary for the analysis of TCR repertoires, we used cap switching technology and employed specific synthesis primers [4] (Fig. 1). Cap switching ensures uniform amplification of fragments that correspond to different T-cell receptors, thus preventing misrepresentation of variable gene segments. Each cDNA library was prepared using the entire amount of total RNA isolated from 3.5 billion mononuclear cells. The obtained total cDNA was used for amplification in full. Sequencing yielded over 30 million reads, the minimum number of reads was 1.3×10^6, the maximum number was 6.5×10^6. Molecular identifiers introduced during the cDNA synthesis stage were used as a filter during the analysis (Fig. 1): only those uniquely labeled cDNA molecules that were covered by at least 3 reads were factored into. The analysis yielded 27,000 to 400,000 read groups each carrying a unique barcode and corresponding to a unique cDNA molecule.

Sequencing data was normalized by random selection of 25,000 cDNA events per sample, i. e. to the size of the smallest dataset obtained from a patient's sample. It means that further analysis was performed on 25,000 unique TCR beta chain cDNA molecules representing each sample. Previously analyzed TCR repertoires of healthy individuals [11] were used as a reference. The reference dataset was also downsized to 25,000 random cDNA events per sample. Our previous experiments demonstrated that one cDNA event is on average equivalent to one T-cell [15]. Thus, the diversity of a T-cell repertoire was inferred from the analysis of 25,000 random peripheral blood T-lymphocytes.

The following metrics were used to estimate the diversity of the TCR beta chain repertoire: the lower bound for species richness (the Chao1 estimator), the Shannon-Wiener index of even distribution and the observed diversity of CDR3 (per 25,000 T-lymphocytes).

No significant differences were found in TCR repertoire profiles between patients with vasculitis and healthy donors (Fig. 2, B–D). But although the proportions of naive T-cells in the peripheral blood of diseased and healthy individuals were almost the same, Chao1 values were lower for patients with vasculitis (Fig. 2, B, E), indicating the depletion of the TCR diversity.

Analysis of beta-chain variable segments

Normally, the role of clonal T-cell populations in pathology is estimated by spectratyping (analysis of CDR3 fragment lengths in general and TCR beta chain V-segments in particular).

A few researchers used TCR spectratyping to demonstrate involvement of clonal and oligoclonal T-cells carrying a particular V-segment in GPA [18] and EGPA [17]. Later, TCR V-segments were found to be overrepresented in the peripheral blood of patients with EGPA in another flow cytometry-based study [23].

These findings inspired the search for the described TCR V-segments in patients with GPA and EGPA, prompting us to analyze the frequencies of different beta-chain segments in the diseased and healthy individuals. We revealed no specific expression patterns of V- or J-segments that could divide TCR repertoires observed in afflicted patients or healthy controls into distinct clusters.

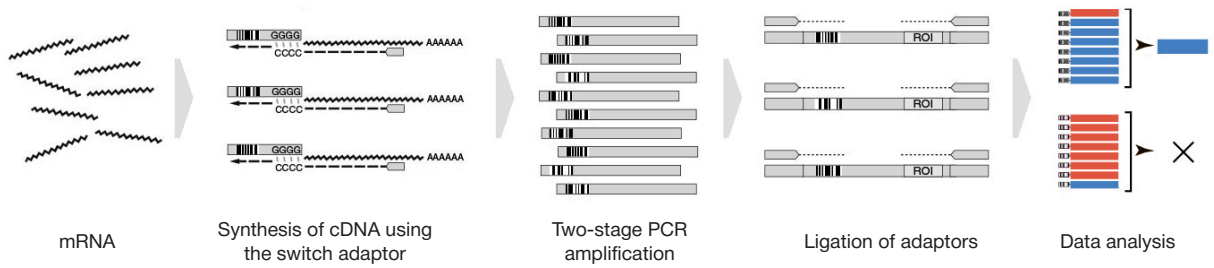

Fig. 1. Preparation of TCR cDNA libraries. The switch adaptor carries a unique sequence (shown as a barcode in the picture) which allows accurate error correction at the stage of data analysis. The amplified fragment carries TCR beta chain CDR3 responsible for receptor diversity

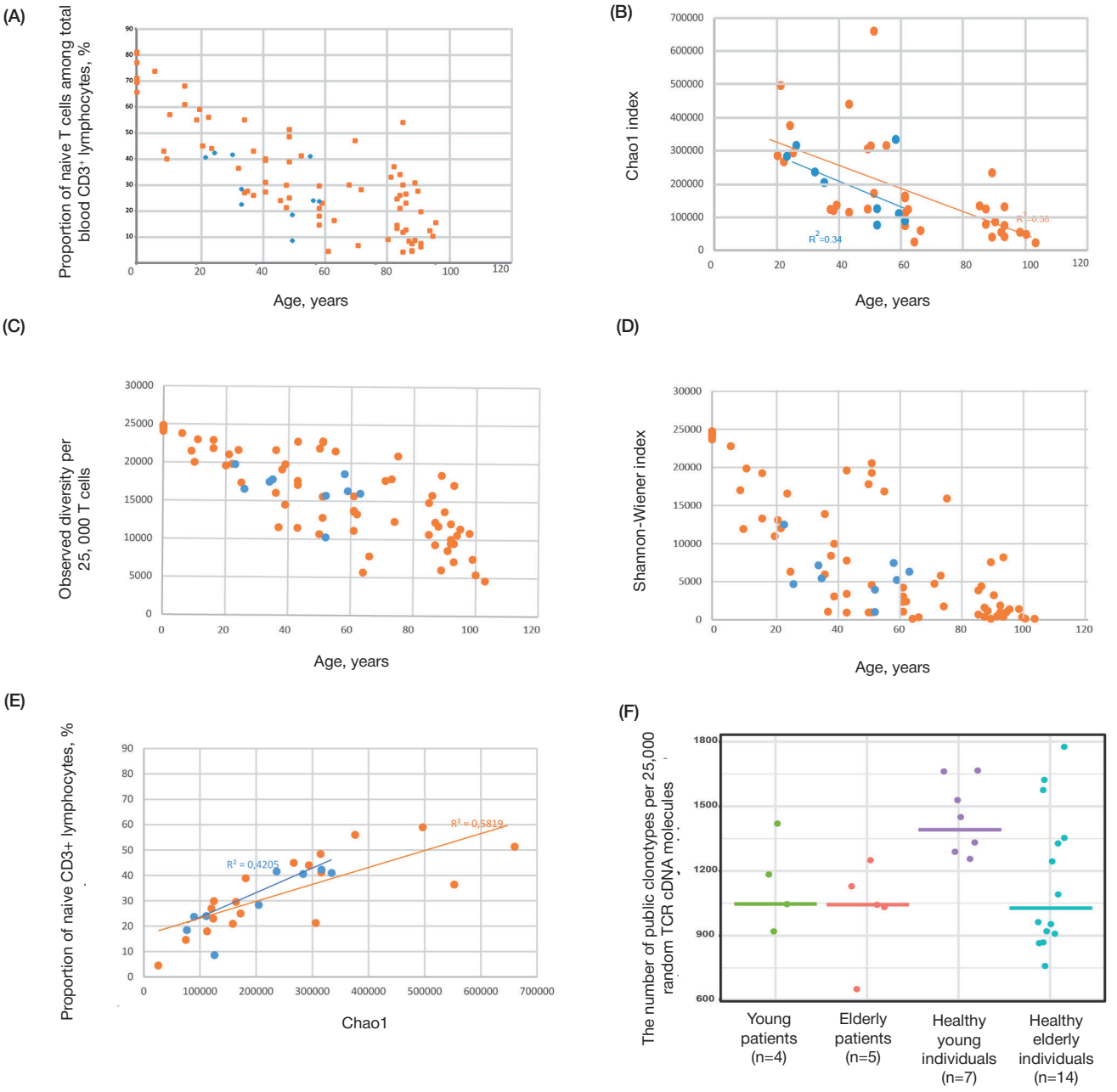

Fig. 2. The proportion of naive T cells among the peripheral blood CD3+ lymphocytes and the diversity of TCR beta chain repertoires in patients with autoimmune vasculitides who previously received high doses of cyclophosphamide (blue dots) and healthy individuals (orange dots) **(A)** The proportion of naive T cells among the total CD3+ lymphocytes plotted against participants' age. **(B)** The diversity of TCR estimated with Chao1. The major contribution is made by low-frequency clones, i.e. TCRs of naive lymphocytes. **(C)** The diversity of CDR3 sequence variants per random 25, 000 TCR beta chain cDNA molecules **(D)** The diversity of TCR beta chains estimated with the Shannon-Wiener index used to measure the evenness of clonotype distribution in the TCR repertoire. **(E)** The proportion of naive T cells among total CD3+ lymphocytes, accounting for Chao1. **(F)** The number of public clonotypes in clone sets depending on donor's age. Fig. B-E are based on the analysis of 25,000 randomly selected TCR beta chain cDNA molecules

Search for GPA- and EGPA-associated CDR3 sequences

Next, we analyzed the sequencing data of our patients for the presence of the annotated CDR3 sequences associated with both GPA and EGPA [17, 18, 23]. We searched in unnormalized clone sets. We did not find any CDR3 motifs previously announced to be GPA — or EGPA —associated.

Estimation of the proportion of public clonotypes in TCR repertoires

High-throughput sequencing of immune repertoires is very instrumental in obtaining individual lists of TCR sequences the organism employs to develop adaptive immune response. Among the millions of TCR variants sequenced by the authors of this work and other researchers, the so-called public (or non-unique) TCR clonotypes have been identified, occurring repeatedly in the immune repertoires of unrelated participants [11, 19, 20]. Identical TCR sequences found in unrelated individuals are thought to be generated through stochastic recombination of TCR gene segments or in an attempt to produce optimal TCR clones for effective immune response to a widespread pathogen or as a result of TCR co-evolving with a persisting infection transmitted through generations. Relatives share more TCR clonotypes than unrelated donors. On the whole, the genetic environment of a

donor cohort can affect the outcome of the analysis of public TCR repertoires.

In our previous work we showed that the proportion of public TCR clonotypes decreases with age [4]. In other words, it is a marker of TCR repertoire ageing. Previously we drew a list of human TCR beta-chain clonotypes present in the T-lymphocyte repertoires of different donors. Public clonotypes were extracted from the sequences of highly diverse TCR repertoires of umbilical cord blood where they are abundant. We selected clonotypes with the identical CDR3 amino acid sequence found in at least 4 of 8 samples of umbilical cord blood obtained from healthy donors. This list of clonotypes was further supplemented with short high-frequency CDR3 variants that are generated during VDJ recombination, primarily during fetal development [16], and sequences without random N-nucleotide insertions at the V–D or D–J junctions resulting from a simple VDJ rearrangement in embryos in the absence of TdT-transferase expression.

Public clonotypes of TCR beta chains emerged in the embryonic stage often coincide with the list of super public TCR variants that can be observed in healthy adults (8 to 85 years, n = 68, super public clones found in 20 samples of 68, data unpublished).

In the course of the analysis, we used clone sets normalized to 25,000 cDNA events. Using a list of 7,200 public clonotypes, we compared their frequency to the frequencies in the reference cohort of healthy individuals and patients with vasculitis, to reveal that younger patients had reliably fewer public clonotypes ($p = 0.04$, Mann–Whitney U, Fig. 2, E) in their TCR repertoires, compared to healthy donors of the same age.

DISCUSSION

As mentioned above, no research works have been published so far dedicated to deep sequencing of TCR repertoires of patients with GPA and EGPA. Sanger sequencing of T-cell receptors has, however, been used to discover a common CDR3 motif among highly represented Vbeta 21 clonotypes of the TCR family in patients with EGPA [17]. Also, there are reports on the Vbeta8 clonotype dominating the TCR repertoire of patients with GPA [18].

In the course of our study, we did not observe any specific clonotypes or expression patterns of V- and J-segments that could be used to discriminate between GPA or EGPA patients and healthy individuals of the same age. Perhaps, we need a larger sample to identify T-cell clones associated with the disease. In the recent study conducted in 191 patients with ankylosing spondylitis and 227 healthy individuals, the consensus TCR beta CDR3 motif was identified to implicate in the pathogenesis of this autoimmune disease [21]. A combination of techniques, such as the analysis of TCR repertoires obtained from the lymphocytes migrating to inflammation sites and a new algorithm for calculating the probability of assembly of a particular CDR3 sequence, may provide a solution to the problem of autoimmune clone identification [22].

The analysis of TCR diversity demonstrated the absence of significant differences in the degree of clonality and the observed CDR3 diversity between patients with vasculitis and healthy controls. Slight variations in the proportion of naive T-lymphocytes and Chao1 values may indicate a minor peripheral expansion of naive clones recovered after CPH treatment.

Our study demonstrates that young patients have a significantly lower number of public clonotypes in comparison with healthy donors of the same age. Such structural changes of the TCR repertoire of young patients after exposure to high doses of CPH may indicate premature of T-cell adaptive immunity. We conclude that the subset of public TCR representing naive T-lymphocytes formed during the embryonic stage is considerably downsized by high doses of CPH. Further research is necessary to demonstrate the long-term effects of such changes.

CONCLUSIONS

Using deep sequencing, we have analyzed TCR repertoires of diseased individuals and healthy donors to discover that TCR repertoires are able to recover after the exposure to high doses of cyclophosphamide. The number of public clonotypes in patients with autoimmune disorders is, however, lower than in healthy individuals, which indicates premature ageing of TCR repertoires.

We have not observed any changes that may be associated with autoimmune vasculitis. Patients' sequencing datasets contained no T-cell clonotypes previously annotated in the literature as implicated in the pathogenesis of the disease.

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ESTIMATING THE NUMBER OF HIV-SPECIFIC T-CELLS IN HEALTHY DONORS USING HIGH-THROUGHPUT SEQUENCING PROFILES OF T-CELL RECEPTOR REPERTOIRES

Eliseev AV, Fedorova AD, Lebedin MY, Chudakov DM, Shugay M ✉

Pirogov Russian National Research Medical University, Moscow, Russia

In-depth study of mechanisms of immune response to the human immunodeficiency virus (HIV) is critical for better understanding of how immunodeficiency develops in patients with HIV, as well as for designing effective immunotherapy strategies and vaccines against the virus. In this work we analyze sequencing profiles of T-cell receptor repertoires previously obtained from healthy donors (601 Americans and 65 Russians) to estimate the population frequency of HIV-specific naive T-cells. We demonstrate that frequencies of T-cells recognizing different HIV epitopes vary considerably across the population (F-statistic = 2007, $p < 10^{-100}$, ANOVA). Although the frequency of T-lymphocytes recognizing a particular epitope does not change significantly between the individuals, it still largely depends on the presence of certain HLA alleles ($p < 0.01$, post-hoc Tukey's test), cytomegalovirus infection ($F = 61$, $p = 7 \times 10^{-15}$, ANOVA), and age (Pearson correlation coefficient ranging from –0.53 to –0.14 in different groups).

Keywords: HIV, T-cell receptor, HLA, CMV, high-throughput sequencing

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✉ **Correspondence should be addressed:** Mikhail Shugay
ul. Miklukho-Maklaya, d. 16/10, Moscow, Russia, 117997; mikhail.shugay@gmail.com

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ОЦЕНКА ЧИСЛА ВИЧ-СПЕЦИФИЧНЫХ Т-ЛИМФОЦИТОВ У ЗДОРОВЫХ ДОНОРОВ ПО ДАННЫМ ВЫСОКОПРОИЗВОДИТЕЛЬНОГО СЕКВЕНИРОВАНИЯ РЕПЕРТУАРОВ Т-КЛЕТОЧНЫХ РЕЦЕПТОРОВ

А. В. Елисеев, А. Д. Федорова, М. Ю. Лебедин, Д. М. Чудаков, М. А. Шугай ✉

Российский национальный исследовательский медицинский университет имени Н. И. Пирогова, Москва

Детальное изучение механизмов формирования иммунного ответа против вируса иммунодефицита человека (ВИЧ) необходимо для лучшего понимания процессов развития иммунодефицита у пациентов с ВИЧ-инфекцией, разработки эффективных методов ее иммунотерапии и новых вакцин против вируса. В исследовании нами были проанализированы данные ранее полученных результатов секвенирования репертуаров Т-клеточных рецепторов для большого числа здоровых индивидуумов (601 донор из американской популяции и 65 доноров из российской популяции) с целью определения частоты наивных Т-лимфоцитов, обладающих специфичностью к ВИЧ, в популяции. Мы обнаружили, что на уровне популяции имеет место значительная вариабельность между различным эпитопами ВИЧ в частотах специфичных Т-клеток (F-statistic = 2007, $p < 10^{-100}$, ANOVA). Несмотря на то, что частота Т-лимфоцитов, специфичных к одному и тому же эпитопу, была достаточно стабильной между индивидуумами, мы обнаружили, что она значительно зависит от таких факторов, как наличие у человека определенных аллелей HLA ($p < 0,01$, пост-хок тест Тьюки), наличие цитомегаловирусной инфекции ($F = 61$, $p = 7 \times 10^{-15}$, ANOVA), а также возраст (коэффициент корреляции Пирсона в диапазоне от –0,53 до –0,14 для разных групп).

Ключевые слова: ВИЧ, Т-клеточный рецептор, HLA, CMV, высокопроизводительное секвенирование

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✉ **Для корреспонденции:** Шугай Михаил Александрович
ул. Миклухо-Маклая, д. 16/10, Москва, 117997; mikhail.shugay@gmail.com

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The highly mutable human immunodeficiency virus (HIV) easily evades the immune system [1]. Still, there is hope for anti-HIV immunotherapy, considering the variety of immunogenic HIV epitopes [2] and protective human leukocyte antigen (HLA) alleles [3], as well as the phenomenon of the so-called elite controllers [4]. Although attempts to develop an HIV vaccine have not paid off yet, the accumulated evidence suggests that the T-cell therapy may potentially be as effective as the conventional antiretroviral treatment [5].

In this work we draw on the assumption that the proportion of antigen-specific T-cells occurring in the naive T-lymphocyte population determines the magnitude of the immune response [6]. Knowing which HIV epitopes tend to be readily recognized by the immune system of a person who carries a particular set of HLA alleles will help to elucidate mechanisms of immune protection against HIV and finally make headway in the development of an HIV vaccine.

The ability of T-cells to recognize foreign antigens is encoded by alpha- and beta-chain genes of T-cell receptors (TCR). Their diversity is incredible: the number of unique sequences (variants) in a person's beta chain is estimated to be over 10^9, while the total number of TCR variants generated in the thymus

gland of each member of the population is almost infinite [7]. Massively parallel sequencing of immune repertoires (RepSeq) has evolved to simultaneously produce millions of TCR reads per studied sample, e. g. of peripheral blood mononuclear cells [8]. Currently existing methods of T-cell sorting, especially those based on MHC multimer staining [9], yield a wealth of information about antigen-specific TCR. In this light, RepSeq can be conveniently used to analyze individual TCR repertoires. For example, data generated by RepSeq can be further annotated *in silico* and the number of epitope-specific T-cells can be estimated using the regularly updated VDJdb repository of TCR sequences with known antigen specificity [10].

That said, it is almost impossible to accurately quantify antigen-specific T-cells in the naive T-cell population using standard techniques, such as flow cytometry. Because the population of T-cells that recognize a particular epitope is often very small (< 1 %) [11], magnetic bead enrichment may be needed [12], which, unfortunately, can distort the results. In contrast, RepSeq reliably reports T-cells with frequencies as low as 0.001 % [13].

Having a large dataset of TCR sequences at our disposal obtained from 65 Russian and 601 American donors and another dataset of 1,688 TCR with known epitope specificity (see Methods), we have attempted to study the frequency of HIV-specific T-cells in the population. The following hypotheses have been tested:

1) frequencies of epitope-specific T-cells in the TCR repertoires of healthy individuals vary considerably depending on the epitope;

2) cytomegalovirus (CMV) infection in the individual affects the proportion of HIV-specific T-cells in his T-cell repertoire;

3) the number of HIV-specific T-cells depends on the presence of specific HLA alleles in the individual;

4) the number of HIV-specific T-cells depends on the individual's age and sex.

METHODS

We analyzed the datasets of sequenced TCR beta chains obtained by Emerson et al. [14] and Britanova et al. [15]. We

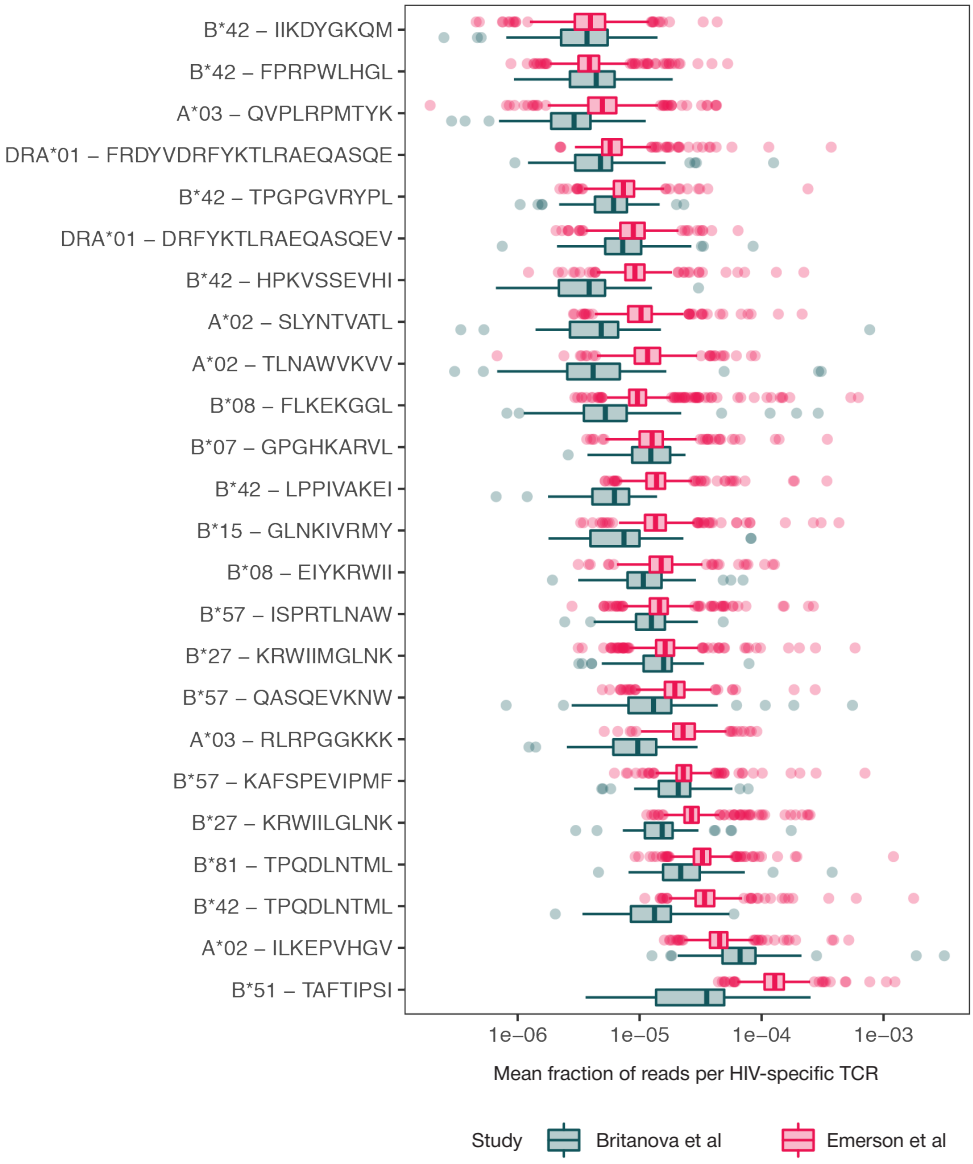

Fig. 1. Mean abundance of different HIV-specific T-cell receptors (TCR) in the T-cell repertoires sequenced by Emerson et al. [14] and Britanova et al. [15]. The X-axis represents distribution of an average-sized clonotypic population derived from an HIV-specific T-cell (fraction of all TCR beta chain reads) in the samples from [14] (n = 65) and [15] (n = 601). The Y-axis represents epitopes for which HIV-specific TCR sequences are known (according to VDJdb); data format: HLA allele : epitope sequence

did not use all of the sequenced data obtained by Emerson, selecting the TCR repertoires of only those donors whose HLA haplotype had been identified and CMV status was known — a total of 601 samples. We also filtered out umbilical cord blood TCR from Britanova et al. study's sample, saving for the analysis only the repertoires of 65 healthy adults. Data preprocessing and segment mapping for sequences borrowed from [15] were performed with MIGEC [16] and MiTCR [17] software tools. Segments from [14] were additionally mapped, V- and J-segment genes were identified and sequencing errors were corrected using MiXCR [18]. Data were cleaned of non-functional sequences containing stop-codons or frameshifts using VDJtools [19].

Annotation, i. e. prediction of HIV-specific TCR, was done using VDJtools/VDJdb-standalone [10]. VDJdb was searched for HIV-specific TCR; epitopes represented in the database by less than 10 TCR variants were excluded from the analysis. A RepSeq TCR was counted as specific to a particular epitope if the amino acid sequence of the epitope's hyper variable CDR3 region differed by no more than 1 substitution from the corresponding TCR sequence stored in VDJdb. This approach yields a substantially larger set of annotated TCR, with only a tiny percent of erroneous annotations, as shown in [10].

Statistical analysis was done with R. The following statistical algorithms were used: ANOVA, Tukey's post hoc test and correlation analysis. Values for the F-statistic, Spearman's rank correlation and Student's p are provided in the Results section.

RESULTS

Frequency estimates obtained by flow cytometry for HIV-specific TCR convincingly demonstrate that the proportion of specific T-cells in the naive (intact) repertoire varies considerably, differing by 1 or 2 orders of magnitude between the epitopes, while remaining fairly stable between different individuals [12]. Analysis of high-throughput TCR sequencing data conducted in the course of our study (Fig. 1) supports these observations: frequencies of HIV-specific TCR have been found to be highly epitope-dependent ($F = 2007$, $p < 10^{-100}$, ANOVA), which, however, bears no connection to the presenting HLA allele ($F = 0.03$, $p = 0.86$, ANOVA). Importantly, there is a significant discrepancy in the estimates for Emerson's and Britanova et al. study's datasets ($F = 1690$, $p < 10^{-100}$; average frequency of HIV-specific TCR is higher for Emerson et al. study's data), which can be explained by different structures of TCR libraries and techniques used for their preparation. Emerson et al. worked with DNA samples employing multiplex PCR, while Britanova et al. used RNA samples, 5'RACE and molecular barcoding [20]. Skipping the details, we will, however, emphasize that molecular barcoding ensures more accurate quantification of TCR in the sample [15].

In the study by Emerson et al. the donors were divided into two cohorts based on their serologic status, i. e on the presence or absence of CMV infection. With sequencing data at our disposal, we seized this opportunity to evaluate

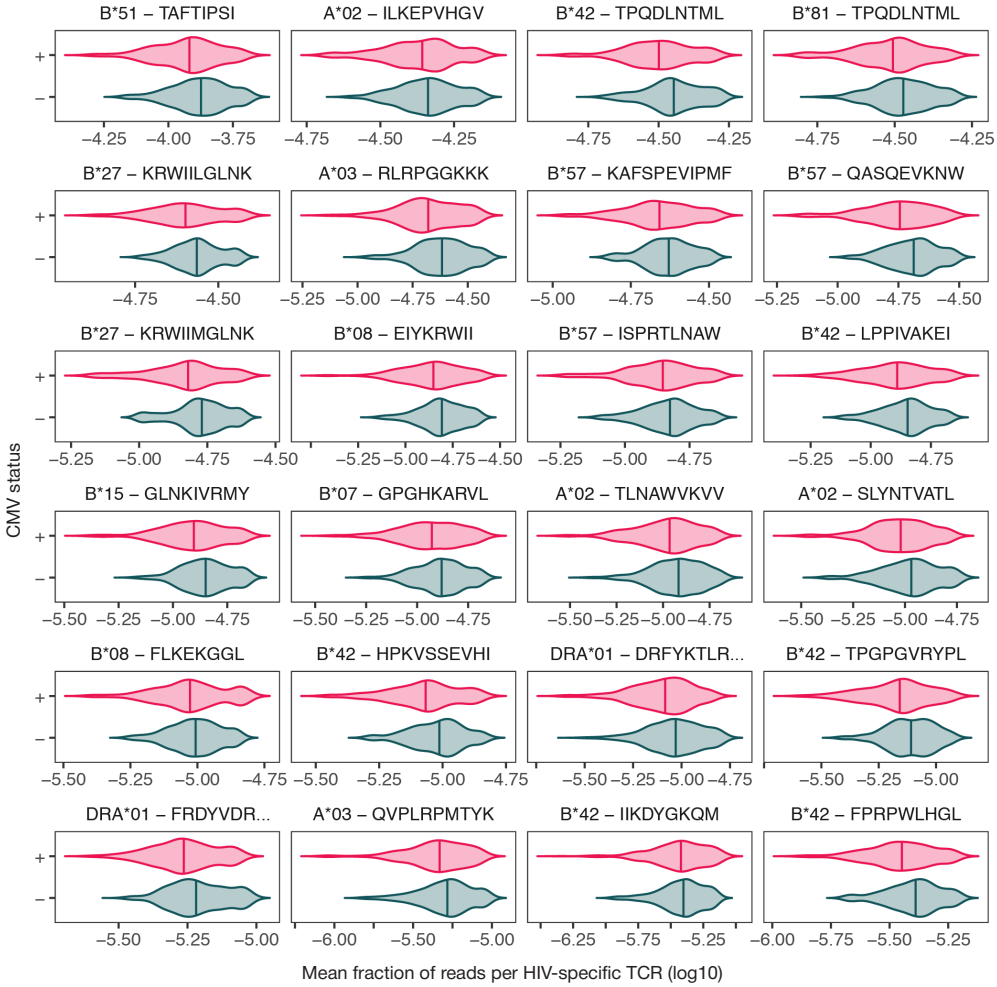

Fig. 2. Abundance of HIV-specific TCR in the sample of individuals with known cytomegalovirus status. Results for CMV-positive (+) and CMV-negative (–) individuals are provided separately for each epitope. Plotted along the X-axis is the common logarithm of an average-sized clonotypic population of an HIV-specific T-cell (fraction of total TCR beta chain reads). Data have been winsorized for graphical clarity: the figure shows samples with values ranging between the 5th and 95th percentiles

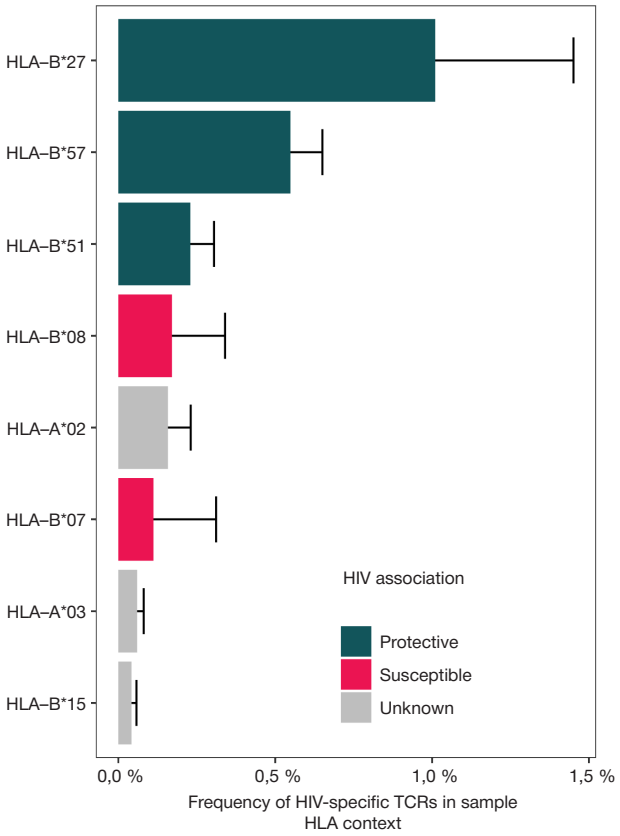

Fig. 3. The total frequency of TCR specific to HIV-epitopes represented by donor's HLA allele sets (data borrowed from Emerson et al.). Plotted along the X-axis is mean percent (for groups of individuals with the studied HLA allele) of the total number of reads for TCR sequences specific to HIV epitopes represented by a specific HLA allele. Standard deviation is provided for each value. The list of alleles was obtained by intersecting datasets from VDJdb and annotations from Emerson et al. study. Allele HLA-B*42 was excluded from the analysis, because it was represented by only one sample. Other alleles were present in more than 35 of 601 donors. Protective alleles and alleles associated with susceptibility to HIV were borrowed from Goulder and Walker's work [3]

the impact of CMV infection on the frequency of HIV-specific TCR in donors' repertoires. As shown in Fig. 2, the frequency of HIV-specific TCR was significantly higher for CMV-negative individuals regardless of the HIV epitope ($F = 495$, $p < 10^{-100}$, ANOVA). Of note, if TCR were not grouped based on the epitope they recognize, i.e. if the epitope-related difference in HIV-specific TCR frequencies was ignored, the result would be far less significant ($F = 61$, $p = 7 \times 10^{-15}$, ANOVA).

Information about the HLA haplotypes of the donors provided by Emerson et al. was used to estimate the number of HIV-specific TCR considering that the donor may have some of HLA alleles capable of representing an HIV epitope. As shown in Fig. 3, the largest proportion of HIV-specific TCR is observed for putatively protective B27, B57 and B51 HLA alleles [3]. For these 3 alleles the number of HIV-specific TCR is significantly higher than for 5 other alleles ($p < 0.01$, Tukey's post hoc test), except for the differences between alleles B51 and B08. It should be noted that we had to recruit a relatively small number of alleles for out study because there were no known HIV epitopes for other alleles in VDJdb.

Age-related changes in the structure of the T-cell repertoire were described in a number of previously published works [21] reporting the reduction of the observed repertoire diversity due to clonal expansions caused by chronic infections. Impoverished diversity results in the decreased proportion of T-cells (including the HIV-specific T-cells) capable of recognizing previously unencountered pathogens (Fig. 4): $R = -0.35$ (Spearman's correlation coefficient here and else where; $p = 0.003$) for the data borrowed from Britanova et al. and $R = -0.20$ ($p = 0.001$) for CMV-negative individuals from Emerson's work. At the same time, CMV-positive patients demonstrate a less pronounced decrease in HIV-specific TCR ($R = -0.14$, $p = 0.03$). Massive clonal expansions (> 5 % of TCR sequences) observed in CMV+-patients from the work by Emerson et al. can be explained by the cross-reactivity of HIV-specific clonotypes to CMV epitopes. Data borrowed from Britanova et al. illustrates reduced levels of HIV-specific T-cells in men ($R = -0.53$, $p = 0.002$) but not in women ($p = 0.22$), because the female cohort included centenarians (aged 80 to 100 years) whose repertoires are typically very specific [15].

DISCUSSION

Our findings indicate that the median frequency of HIV-specific T-cells in healthy individuals can vary by 1 or 2 orders of magnitude depending on the studied HIV epitope, which is consistent with estimates obtained by other researchers who used different techniques [11, 12, 22]. A far less pronounced variability is observed between the individuals for the frequencies of T-cells recognizing a particular epitope.

It should be noted that one of the major factors determining the frequency of a particular TCR sequence in the population is probability of its assembly during V(D)J recombination [7]. This process is well described by existing statistical models [23]. Recombination parameters do not vary a lot between across the population, which is consistent with our findings. Therefore, we infer that frequencies of HIV-specific T-cells calculated *in silico* are an important and reliable parameter of the magnitude of the immune response to particular epitopes at the population level.

To sum up, development of effective vaccination strategies should account for the pool of epitopes that may be represented in the individual HLA context and for the proportion of naive T-cells capable of recognizing these epitopes.

Frequencies of HIV-specific T-cells studied in the context of known HLAs vary significantly depending on the HLA. The highest frequencies of HIV-specific TCR were observed for protective alleles listed in [23].

Besides, we noticed that the proportion of HIV-specific T-cells goes down with age in individuals with CMV infection. These data are consistent with broader observations of the dynamics of T-cell repertoire diversity affected by age and chronic infections, including CMV [24–26]. Sex-based comparison of sequencing data borrowed from Britanova et al. and Emerson et al. works returns controversial results, because Britanova et al. study recruited female centenarians.

CONCLUSIONS

Our work demonstrates that sequencing of immune repertoires and the subsequent bioinformatic analysis allow in-depth study of antigen-specific T-cell populations. RepSeq is a valuable tool for estimating the frequency of HIV-specific T-cells in the repertoires of healthy donors that can be used to identify the factors affecting this frequency. As the VDJdb will grow to include more annotated sequences, our data will be supplemented with new HIV epitopes and HLA alleles. In the future, exploration of cell repertoires of HIV-infected donors carrying different HLA alleles, including elite controllers, will help us to identify those HIV-specific TCR present in the naive T-lymphocyte populations that have higher frequency and are associated with HIV inhibition.

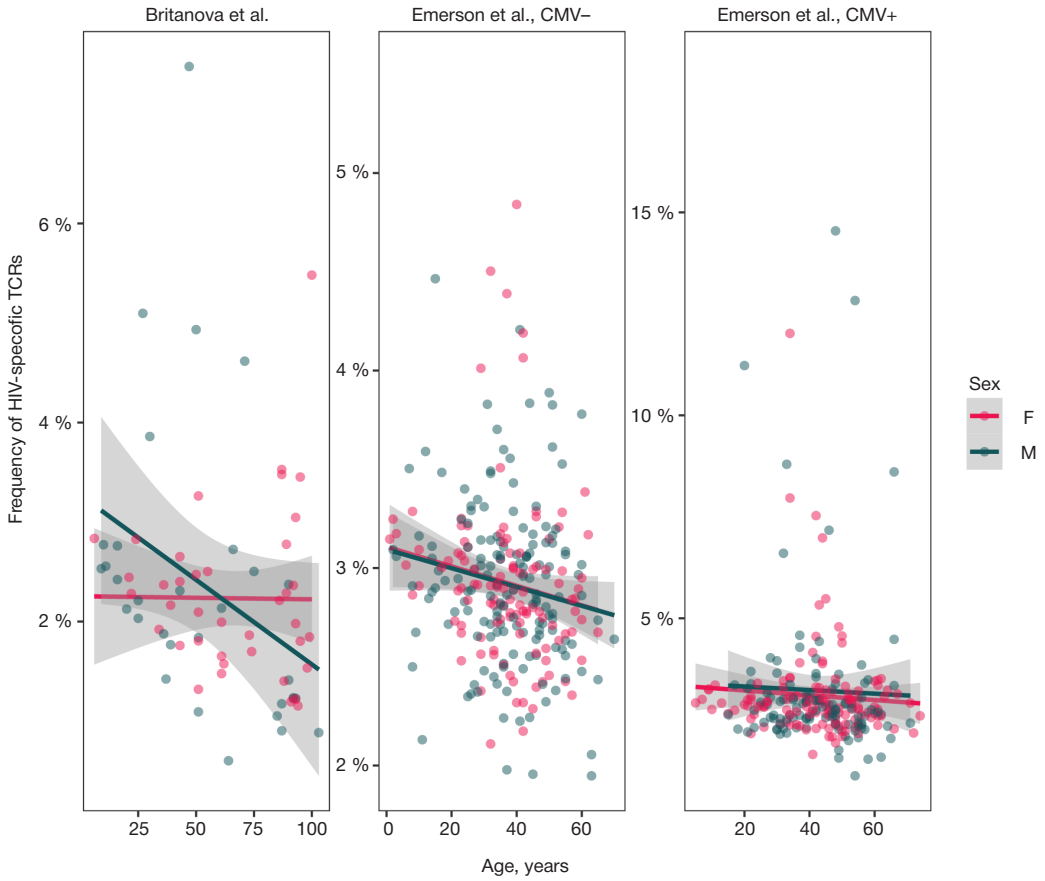

Fig. 4. Changes in the total frequency of HIV-specific TCR in donors of different sex and age. Data from Emerson et al. are distributed into groups based on the CMV status of the participants. The graph also shows results of linear modeling for HIV-specific TCR dependence on age for males and females

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CLINICAL CHARACTERISTICS OF PREMENSTRUAL PAINS

Dobrokhotova YuE[1], Friptu VG[2], Grishin II[1], Saprykina LV[1], Mazur IA[1,2,3] ✉

[1] Department of Obstetrics and Gynecology, Faculty of General Medicine,
Pirogov Russian National Research Medical University, Moscow, Russia

[2] Department of Obstetrics and Gynecology,
Nicolae Testemiţanu State University of Medicine and Pharmacy, Chisinau, Republic of Moldova

[3] Medsi Group, Moscow, Russia

Premenstrual syndrome (PMS) profoundly affects a woman's quality of life, causing physical and emotional distress. This study aimed to describe premenstrual pains in reproductive-age women (18–45 years). The main group included 136 women with moderate and severe PMS; the control group consisted of 136 healthy females with only sporadic premenstrual symptoms. We encouraged the participants to rate their symptoms using the menstrual distress questionnaire by Rudolf H. Moos and to keep a symptom diary over the course of 3 menstrual cycles. We also used the visual analogue scale, which allows estimating pain intensity. In the main groups the participants scored an average of 47.14 ± 3.67 total points on the distress questionnaire (moderate PMS), whereas the controls scored 10.28 ± 1.94 points (mild PMS) ($p < 0.05$). Among the most typical premenstrual symptoms observed in the main group and the controls were: headaches (66.17 % vs. 22.79 %, respectively; $p < 0.001$); breast tenderness/pain (83.08 % vs. 49.26 %, respectively; $p < 0.001$); pelvic pain (70.58 % vs. 35.29 %, respectively; $p < 0.001$); bloatedness/stomach ache (64.7 % vs. 25.73 %, respectively; $p < 0.001$), and muscle/joint pain (51.47 % vs. 21.32 %, respectively; $p < 0.001$). The average number of premenstrual symptoms observed in the main group was 5.62 ± 0.92, of which 2.47 ± 0.68 represented intense pains determining PMS severity. The results of our study suggest that premenstrual symptoms should be monitored prospectively over at least 2 consecutive menstrual cycles using a diary, because retrospective data are unreliable.

Keywords: menses, premenstrual syndrome, premenstrual symptom, pain, pain symptome, menstrual distress questionnaire, symptom diary

✉ **Correspondence should be addressed:** Inna Mazur
ul. Yuzhnaya 14, kv.181, Drozhzhino, Leninsky district, Moscow region, 142718; mazur_ina@mail.ru

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КЛИНИЧЕСКИЕ ОСОБЕННОСТИ БОЛЕВЫХ СИМПТОМОВ ПРИ ПРЕДМЕНСТРУАЛЬНОМ СИНДРОМЕ

Ю. Э. Доброхотова[1], В. Г. Фрипту[2], И. И. Гришин[1], Л. В.Сапрыкина[1], И. А. Мазур[1,2,3] ✉

[1] Кафедра акушерства и гинекологии, лечебный факультет,
Российский национальный исследовательский медицинский университет имени Н. И. Пирогова, Москва

[2] Кафедра акушерства и гинекологии,
Государственный университет медицины и фармации имени Н. Тестемицану, Кишенев, Молодова

[3] ГК «Медси», Москва

Предменструальный синдром (ПМС) — патология, которая может значительно ухудшать качество жизни женщины, влияя на ее физическое и эмоциональное состояние. Целью исследования являлось определение особенностей болевых проявлений при ПМС у женщин репродуктивного возраста (18–45 лет). В основную группу включили 136 женщин с ПМС средней/тяжелой степени, в контрольную — 136 здоровых женщин с единичными предменструальными симптомами. Для оценки болевых проявлений использовали Менструальный дистресс-опросник Рудольфа Муса, менструальный дневник (в течение 3 последовательных циклов) и визуальную аналоговую шкалу (позволяет оценить интенсивность болей). Средняя общая оценка по дистресс-опроснику в основной группе составила 47,14 ± 3,67 балла (ПМС средней тяжести), а в контрольной — 10,28 ± 1,94 балла (ПМС легкой степени) ($p < 0,05$). Среди болевых предменструальных симптомов встречались: головные боли — в 66,17 % случаев в основной группе и в 22,79 % случаев в контрольной группе ($p < 0,001$); тяжесть/боль в молочных железах — в 83,08 % и 49,26 % случаев ($p < 0,001$); тазовые боли — в 70,58 % и 35,29 % случаев ($p < 0,001$); вздутие/боли в животе — в 64,7 % и 25,73 % случаев ($p < 0,001$); боли в мышцах и суставах — в 51,47 % и 21,32 % случаев соответственно ($p < 0,001$). В среднем в основной группе у пациенток отмечали 5,62 ± 0,92 предменструальных симптома, из них 2,47 ± 0,68 были болевыми выраженной интенсивности, определяя степень тяжести ПМС. Полученные результаты указывают на то, что симптомы ПМС следует подтверждать проспективными ежедневными оценками в течение не менее 2 последовательных циклов, т. к. ретроспективный анамнез не является достаточно надежным.

Ключевые слова: менструация, предменструальный синдром, предменструальный симптом, боль, болевой симптом, менструальный дистресс-опросник, менструальный дневник

✉ **Для корреспонденции:** Мазур Инна Александровна
ул. Южная, д. 14, кв. 181, д. Дрожжино, Ленинский р-н, Московская область, 142718; mazur_ina@mail.ru

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Premenstrual syndrome (PMS) is a pathology associated with the menstrual cycle. PMS adversely affects the physical and emotional state of women of childbearing age; there is a variety of related somatic and psychoemotional symptoms that express during the luteal phase of menstrual cycle and disappear when menstruation begins [1].

Russian researchers identify four forms of PMS: neuropsychiatric, cephalic, edematous and crisis. Manifestations of the neuropsychiatric form include irritability, depression, weakness, aggression; those of the edematous form are severity and pain in mammary glands, edema of upper and lower extremities and face, flatulence, abdominal pain, sweating; manifestations of the cephalic form are severe headache, nausea, vomiting, depression, chest pain, sweating; and the crisis form calls forth sympathetic attacks (hypertension, chest pain, tachycardia) that occur in the evening or night and can be triggered by stress, overstrain. Besides, there are atypical forms of PMS: vegetative-ovarian cardiomyopathy, hyperthermia, ophthalmoplegia, cyclic allergic reactions [2].

In Europe, one of the most popular PMS classifications is that developed by the Royal College of Obstetricians and Gynecologists (UK). According to this classification, PMS is a combination of neuropsychiatric and somatic symptoms. Domination of some of these symptoms allows identifying neuropsychiatric, somatic and mixed forms of premenstrual syndrome [3].

Many researchers believe that pain always accompanies PMS, and that pain affects women from both clinical and social viewpoints: it determines severity of the syndrome, their psycho-emotional and general state, influences their behavior in the family and at work, decreases their working efficiency [4]. Pain syndromes most often associated with PMS include: headache (migraine) — 50–86 % of cases, mastalgia — 85–96 %, pelvic pains and abdominal pains — 63–80 %, joints and heart pain — 15–17 % [5].

Besides clinical forms of PMS, there are 3 degrees of its severity: mild, moderate and severe. Mild degree of PMS means there are 1–4 low intensity symptoms manifesting that require no treatment; moderate/severe degrees cause manifestation of a number of pain symptoms accompanied by vegetative and affective symptoms (5 to 12 of them), quite noticeable and significantly worsening the woman's condition and requiring treatment [6].

This study aims to identify clinical characteristics of pain associated with PMS in women of reproductive age.

METHODS

The study was conducted in 2010–2013. 272 women took part in it: 136 of them suffered from moderate to severe PMS (treatment group) 136 more experienced PMS only occasionally (control group). The inclusion criteria were reproductive age (18–45 years old), regular menstrual cycle (25–35 days, 3–7 days of menstruation), no combined oral contraceptives taken, no pregnancy or breastfeeding, no organic pathologies of reproductive and/or nervous systems, no mental illnesses.

Diagnostic criteria accepted by the international medical community were applied to diagnose PMS [3, 6, 7]. Outpatient records, personal questionnaires (age, social status, anamnesis, the nature of menstrual function, reproductive function indicators) and special questionnaires allowed assessing clinical characteristics of pain symptoms and medical and social particularities of participants of the study.

The researchers make extensive use of the Menstrual Distress Questionnaire (MDQ, R. Moos) [8] that consists of 8 clusters uniting 47 symptoms. The symptoms listed in this questionnaire reveal the clinical picture and allow determining dominant premenstrual symptoms (vegetative, endocrine and emotional). The participants filled the questionnaire during the luteal phase of their cycles, when the symptoms' manifestations were maximal.

They kept menstrual diaries for 3 consecutive menstrual cycles; notes contained therein allowed assessing clinical nature and timing of the symptoms' manifestations.

The intensity of pain was assessed with the help of the visual analogue scale (VAS) [9]: 0 points — no pain, 1–3 — mild pain, 4–6 — moderate pain, 7–9 — intense pain, 10 points — very severe pain.

Clinical examination included general and gynecological examination and ultrasound examination of pelvic organs and mammary glands. Additionally, the researchers conducted oncocytological examination of cervix and microscopic examination of vaginal discharge. The above studies aimed to reveal the state of reproductive system organs and find organic pathologies of that system, if any, that could stimulate development or magnification of premenstrual symptoms.

Statistica 7.0 (StatSoft, USA) software was used for statistical processing of the data acquired. Average values were calculated for the indicators studied. Student t-test allowed determining reliability of differences seen between groups ($p < 0.05$).

The study got the approval of the Research Ethics Committee of the Nicolae Testemitanu State Medicine and Pharmacy University, Republic of Moldova (30.03.2009). All patients voluntarily signed the informed consent forms.

RESULTS

Patients in the study groups were comparable in age, physique, menstrual cycle parameters.

Table 1. Rudolph Moss's Menstrual Distress Questionnaire Scores

Cluster of symptoms	Treatment group (n = 136)	Control group (n = 136)	p-value
Pain	8.66 ± 1.43	2.12 ± 1.08	< 0.001
Concentration	6.25 ± 1.17	1.03 ± 0.65	< 0.001
Behavioral change	5.6 ± 1.32	0.78 ± 0.47	< 0.001
Autonomic reactions	4.39 ± 1.47	0.83 ± 0.48	< 0.05
Water retention	4.14 ± 1.11	0.96 ± 0.48	< 0.01
Negative affect	9.11 ± 1.71	1.59 ± 1.03	< 0.001
Arousal	3.87 ± 1.13	2.22 ± 0.98	> 0.05
Control	6.08 ± 1.64	1.0 ± 0.64	< 0.01
Total scores	47.14 ± 3.67	10.28 ± 1.94	< 0.001

No pathologies that could affect PMS symptoms were revealed through oncocytological examination of cervix, microscopic examination of vaginal discharge, ultrasound of pelvic organs and mammary glands.

Analyzing anamneses of the patients, we detected a number of gynecopathies that were equally distributed through the groups, i.e. there were no statistically significant differences in their occurrence there ($p > 0.05$). Inflammatory diseases of pelvic organs (treated earlier) were seen in 36.02 ± 4.11 % cases in the treatment group and 27.94 ± 3.84 % cases in the control group. Ovarian cysts, previously found and treated either conservatively or operatively, were found in 5.14 ± 1.89 and 8.08 ± 2.33 % cases, respectively; uterine fibroids — in 6.61 ± 2.13 and 4.41 ± 1.76 % cases. 7.35 ± 2.23 and 5.88 ± 2.01 % of patients in treatment group and control groups, respectively, underwent gynecologic surgeries (cystectomy, ectopic pregnancy, conservative myomectomy). The frequency of menstrual cycle irregularities (menorrhagia, oligomenorrhea) did not exceed 5 % in both groups.

Filling the Rudolph Moss's Menstrual Distress Questionnaire, patients from the treatment group scored higher than control group patients in all clusters except for the Arousal cluster (Table 1). The average treatment group's score was 47.14 ± 3.67 points, which describes their PMS as moderate (22–51 points). That in the control group was 10.28 ± 1.94 points, i. e. they only suffered from mild PMS (4–21 points).

PMS pains and their reported occurrence were as follows: headaches — 66.17 % of patients in treatment group, 22.79 % of patients in the control group ($p < 0.001$); severity and pain in mammary glands — in 83.08 % and 49.26 % of patients, respectively, ($p < 0.001$); pelvic pains — in 70.58 % and 35.29 % of cases, respectively ($p < 0,001$); swelling/abdominal pains — in 64.7 % and 25.73 % of cases ($p < 0,001$); muscle and joints pain — in 51.47 % and 21.32 % of cases, respectively ($p < 0.001$) (Fig. 1).

It is important to note that the abovementioned PMS pains manifest in combination with emotional and autonomic symptoms. In the control group, the manifestations were rare and their degree mild. In the treatment group, the average number of PMS symptoms manifested was 5.62 ± 0.92, their intensity varied. Of those symptoms, 2.47 ± 0.68 were intensely painful and thus produced a negative effect on the general state and behavior of women (interpersonal relationships and ability to work) (Table 2).

In the treatment group, the symptoms expressed themselves for 7.14 ± 1.0 days per month (5–7 days — 61.03 %, > 7 days — 38.97 %). In the control group, the figure was 2.3 ± 1.28 days per month (1–4 days — 88.24 %, no PMS symptoms — 10.29 %) ($p < 0.01$) (Fig. 2).

Assessing painful symptoms, the researchers took into account frequency, duration of their expression, intensity. The following table presents the data describing clinical parameters of painful PMS symptoms in patients that participated in the study (Table 3).

Headache is one of the symptoms that determine the severity of PMS, prevents women from working efficiently and worsens the quality of their lives. Study participants from the treatment group had more severe and longer lasting headaches than those from the control group. The scores were taken with VAS; the difference was statistically significant ($p < 0.01$) (Table 3). In 36.03 % (n = 49) of them, headache was unilateral, in 41.11 % (n = 37), it was pulsating; 31.11 % (n = 28) had their headaches accompanied by nausea/vomiting, 14.44 % (n = 13) — by lacrimation; 54.44 % (n = 49) suffered from additional acousticophobia and photophobia, 31.11 % (n = 28) reported anxiety; 37.78 % (n = 34) felt drowsy at the same time, and 38.89 % (n = 35) found difficulties concentrating their attention while suffering from headaches; physical capabilities worsened in 32.22 % (n = 29) of patients attacked by a headache, and 42.22 % (n = 38) could not work efficiently.

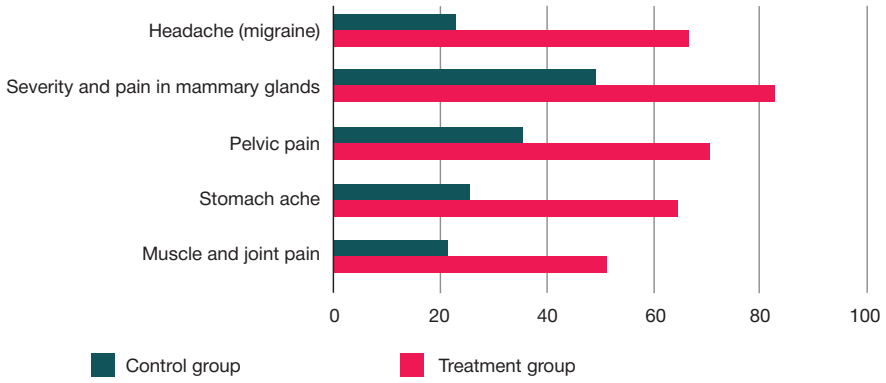

Fig. 1. Frequency of PMS pains suffered by the participants, %

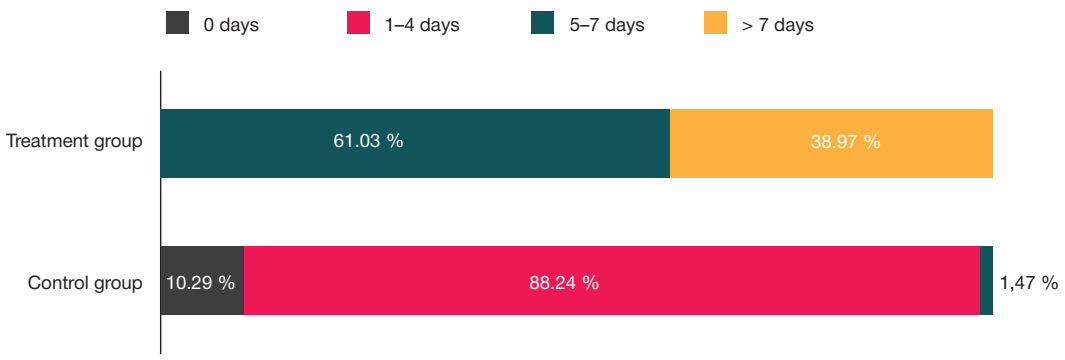

Fig. 2. Duration of clinical manifestation of PMS symptoms, days per month

Table 2. PMS symptoms

Parameter	Treatment group (n = 136)	Control group (n = 136)	p-value
Number of PMS symptoms	5.62 ± 0.92	2.43 ± 1.15	< 0.05
Number of painful PMS symptoms	2.55 ± 0.67	0.9 ± 0.49	< 0.05

Table 3. Painful symptoms parameters

Cluster of symptoms	Treatment group (n = 136)	Control group (n = 136)	p-value
Headache (migraine) duration, days per month intensity, VAS	 5.95 ± 1.36 6.0 ± 1.2	 2.05 ± 0.36 2.1 ± 0.31	 < 0.01 < 0.01
Severity and pain in mammary glands duration, days per month intensity, VAS	 7.77 ± 1.22 5.03 ± 1.03	 3.25 ± 0.97 1.96 ± 0.48	 < 0.01 < 0.01
Pelvic pain duration, days per month intensity, VAS	 4.67 ± 0.94 4.87 ± 0.65	 1.98 ± 0.56 1.86 ± 0.67	 < 0.05 < 0.001
Stomach ache duration, days per month intensity, VAS	 3.91 ± 0.98 3.19 ± 0.58	 1.17 ± 0.17 1.28 ± 0.21	 < 0.01 < 0.01
Muscle and joint pain duration, days per month intensity, VAS	 4.38 ± 0.93 3.73 ± 0.65	 1.34 ± 0.37 1.48 ± 0.45	 < 0.01 < 0.01

Headaches were not migraineous and did not meet the criteria for a migraine aura attack [10].

Severity and pain in mammary glands were reported by 83.08 ± 3.21 % of patients (n = 113) of the treatment group. The duration and intensity of these symptoms were greater than those recorded by the control group (VAS scores; statistically significant difference, $p < 0.01$). As for the pelvic pain, in treatment group it was reported by 70.58 ± 3.9 % of patients (n = 96). Its duration and intensity were greater than those registered in the control group ($p < 0.01$). Often, pelvic pain came together with visceral pain, but its duration and intensity were significantly less notable. Besides, patients have reported muscle and joint pains, which were most often companions to other painful symptoms (Table 3).

Patients of the treatment group reported that painful premenstrual symptoms produced a negative effect on their emotional and general state and quality of life. In the control group, only sporadical expressions of symptoms (mild intensity) were observed, and they did not adversely affect emotional and general state of women.

VAS allowed charting the "pain profile" (Fig. 3) that includes the most common painful PMS symptoms and emotional impact thereof. As for the intensity of those symptoms, they never went above moderate.

DISCUSSION

Due to the variety of clinical manifestations and prevalence of certain symptoms, very often patients suffering from PMS seek medical help from various doctors (therapist, neurologist, endocrinologist), and that help falls short of their expectations. Since PMS is a state ruled by hormones, it is a gynecologist that should be the doctor of choice. If PMS is severe, the team of specialists may be extended to include endocrinologist, neurologist, therapist, etc., depending on the dominating symptoms [11, 12]. We aimed to research characteristics of painful PMS manifestations in order to better diagnose it and optimize treatment offered to PMS patients. The results of our study confirm the hypothesis that painful symptoms express themselves together with psychoemotional and vegetovascular

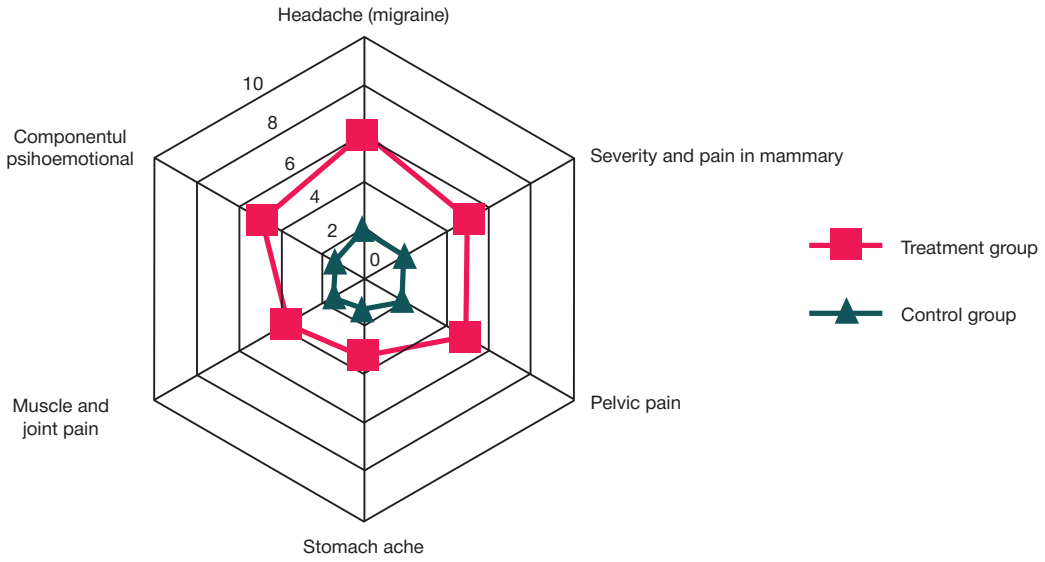

Fig. 3. "Pain profile" of study participants

disorders, which largely determines the severity of clinical manifestations of PMS.

Assessing the state of women suffering from PMS, practitioners should register symptoms prospectively, during 2 or 3 consecutive cycles, with the help of a symptoms diary. Questionnaires and diaries used in the context of this study allowed revealing key features of premenstrual symptoms, including the cyclicity of manifestation exclusively in the premenstrual period. Also, these tools helped identify dominant symptoms and assess their severity.

To a certain extent, the results of this study bring together the opinions various authors have on the clinical manifestations of PMS. According to M. N. Kuznetsova, almost all forms of PMS — cephalal, edematic, and crisis — manifest with one or more painful symptoms [2]. On the other hand, according to the classification by the Royal College of Obstetricians and Gynecologists, PMS manifests through neuropsychiatric, somatic and mixed symptoms [3], and the somatic cluster of PMS symptoms includes neurovegetative and endocrine-metabolic manifestations that have pain (localization varies) as one of the most important constituents. This classification may be more convenient for practitioners, since domination of neuropsychiatric symptoms in PMS means fundamentally different forms of treatment from those prescribed for patients with a predominance of somatic manifestations.

CONCLUSIONS

All in all, PMS manifested itself as a complex of emotional and somatic symptoms (an average of 5.62 ± 0.92 cyclic symptoms). Most often, they were pains of various localization (2.47 ± 0.68 of the total number of symptoms). As reported on a VAS, the duration and intensity of pain in the participants of the study were moderate. Pain symptoms were dominant; they determined the severity of PMS and negatively affected general condition of women.

The results of this study suggest that PMS symptoms should be confirmed with prospective daily assessments made for at least 2 consecutive cycles, since retrospective chart review is not sufficiently reliable.

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MODERN ASPECTS OF MULTIMODALITY APPROACH TO THE DIAGNOSIS OF IDIOPATHIC EPIRETINAL MEMBRANE

Takhchidi KhP[1], Kachalina GF[1], Kasmynina TA[1], Tebina EP[2] ✉

[1] Research Center for Ophthalmology,
Pirogov Russian National Research Medical University, Moscow, Russia

[2] Department of Ophthalmology, Faculty of Pediatrics,
Pirogov Russian National Research Medical University, Moscow, Russia

Idiopathic epiretinal membrane (iERM) is the most common abnormality of the vitreoretinal interface. This condition often stays asymptomatic for a long time. At present, the diagnostic "gold standard" for iERM is spectral-domain optical coherence tomography (SDOCT) and biomicroscopy. However, other diagnostic approaches to ocular fundus pathologies have emerged recently, including multispectral imaging in the MultiColor mode used to estimate tissue proliferation, En Face OCT-angiography that can precisely locate retinal lesions, and microperimetry instrumental in assessing retinal sensitivity and the impact of tissue proliferation. In this work we evaluate the effectiveness of the multimodal approach to iERM diagnosis. We examined 46 patients (46 eyes; mean age was 65.3 ± 11.2 years) with different stages of iERM, pseudophakia and incipient cataract. The multimodal approach allowed us to better discriminate between disease stages and to identify 15 patients with stage 0 iERM, 19 patients with stage 1, and 12 — with stage 2. We were also able to generate a map of the vitreoretinal interface for 2 patients with stage 2 iERM that facilitated the choice of treatment and allowed planning a sparing surgical intervention. Based on our clinical experience and study findings, we conclude that the multimodality approach should be promoted in the clinical setting.

Keywords: multimodality imaging, epiretinal membrane, optical coherence tomography, OCTA En Face, microperimetry, MultiColor

✉ **Correspondence should be addressed:** Ekaterina Tebina
Volokolamskoe shosse, d. 30, korp. 2, Moscow, Russia, 125310; ekaterinatebina@mail.ru

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СОВРЕМЕННЫЕ АСПЕКТЫ МУЛЬТИМОДАЛЬНОЙ ДИАГНОСТИКИ ИДИОПАТИЧЕСКОЙ ЭПИРЕТИНАЛЬНОЙ МЕМБРАНЫ

Х. П. Тахчиди[1], Г. Ф. Качалина[1], Т. А. Касмынина[1], Е. П. Тебина[2] ✉

[1] Научно-исследовательский центр офтальмологии,
Российский национальный исследовательский медицинский университет имени Н. И. Пирогова, Москва

[2] Кафедра офтальмологии, педиатрический факультет,
Российский национальный исследовательский медицинский университет имени Н. И. Пирогова, Москва

Идиопатическая эпиретинальная мембрана (иЭРМ) — наиболее распространенная патология витреоретинального интерфейса. Для этой патологии характерно бессимптомное течение на протяжении достаточно длительного времени. «Золотым стандартом» ее диагностики является исследование с помощью спектральной оптической когерентной томографии (СОКТ) и офтальмобиомикроскопия. Однако к настоящему времени разработаны и другие подходы к выявлению патологий глазного дна, обладающие важными достоинствами: мультиспектральное исследование в режиме MultiColor (оценка распространенности пролиферативного процесса), ОКТ-ангиография в режиме En Face (точная локализация повреждений ретинальных слоев), компьютерная микропериметрия (оценка качества зрения и влияния на него пролиферативного процесса). В исследовании оценивалась эффективность мультимодального подхода в диагностике иЭРМ. Обследовали 46 пациентов (46 глаз; средний возраст — 65,3 ± 11,2 года) с иЭРМ различных стадий, артифакией и начальной катарактой. По результатам обследования у 15 пациентов установили иЭРМ стадии 0, у 19 — стадии 1, у 12 — стадии 2. Применение мультимодального подхода позволило точнее дифференцировать стадии заболевания, а в группе пациентов с иЭРМ стадии 2 также в 2 случаях — разработать карту витреоретинального интерфейса для выбора тактики лечения и плана малотравматичного для сетчатки хирургического вмешательства. Основываясь на собственном клиническом опыте и результатах исследования, мы полагаем, что мультимодальный подход перспективен для более широкого распространения в клинической практике офтальмологов.

Ключевые слова: мультимодальная диагностика, эпиретинальная мембрана, оптическая когерентная томография, En Face ОКТА, микропериметрия, MultiColor

✉ **Для корреспонденции:** Тебина Екатерина Павловна
Волоколамское шоссе, д. 30, корп. 2, г. Москва, 125310; ekaterinatebina@mail.ru

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Idiopathic epiretinal membrane (iERM) is a thin translucent fibrocellular tissue in the center of retina that is capable of contraction leading to distortion of retinal interface surface [1]. This pathology progresses slowly: most often, iERM remains anatomically stable for a long time and its symptoms do not manifest themselves [2]. According to Feng et al. [3], in 25.7 % of cases iERM regresses and in 38.8 % of cases it becomes stable. However, other researchers [4, 5] state that 28.6 %

of iERM cases result in progression to the contractile phase, proliferation on the retina surface that may cause complications like macular edema and macular rupture [6].

Back in 1976, Gass developed an ERM classification; later, Klein modified it based on the color pictures of the fundus [7]:

– stage 0: the membrane is translucent, there is no deformation on the surface of the retina (maculopathy);

– stage 1: uneven wrinkling of retina's inner surface (macular cellophane);

– stage 2: dense membrane on the surface of the retina, general shrinkage of the macula throughout its depth, possibly - macular edema, small hemorrhages, a cotton-like exudate (macular pucker).

This classification is still used in epiretinal fibrosis diagnostics.

To reveal fundus pathologies, including iERM, practitioners routinely make use of ophthalmoscopy, B-mode ultrasound imaging and spectral optical coherence tomography (SOCT) [8]. B-mode ultrasound imaging is the most common non-invasive diagnostics technique applied to fundus, with iERM it does not provide sufficient amount of data. The most effective approach is SOCT. It highlights ERM as a hyper-reflective band adjacent and/or soldered to the inner surface of the retina. In some cases, there are point joints between ERM and retina surface [2]. Besides, SOCT reveals foveolar profile, an important diagnostic indicator. At the beginning, the profile is usually unchanged, but as the pathology progresses, it can be deformed (smoothed out) or disappear altogether [9]. Non-invasiveness, painlessness and quickness make this examination method comfortable for the patient, and its simplicity and technical features -- suitable for screening [10]. Nevertheless, the "golden standard" does not draw a complete picture of structural changes happening in the vitreoretinal interface, as well as that of the pathological process dynamics, its spread and localization and concomitant vision disorders.

Confocal scanning systems allow a more detailed analysis of all layers of the retina and vitreoretinal interface. Basics of ophthalmochromoscopy [11] fostered the development of multispectral laser scanning of the retina (MultiColor), which allows estimating the degree of proliferation on the retinal interface surface intra vitam. The method implies adding 3 images using monochromatic filters: blue (BR; 488 nm), green (GR; 515 nm) and infrared (IR; 820 nm). With MultiColor, surface of the examined membrane and folds thereon appear yellow-green, the intensity of the color depends on elevation of ERM relative to the retina. Blue and green filters allow getting a detailed view of the retina's inner surface and vitreoretinal interface. Combined, they allow finding boundaries of the membrane, folded area and traction component, if any [12]. Multispectral examination is a good complement to SOCT. To increase the effectiveness of screening, these methods can be applied together [9].

Another promising technology is OCT-angiography (OCTA) [13], which allows assessing the state of retinal vasculature and frontal surface of vitreomacular interface (En Face mode) without intravenous administration of contrast agents [14]. The En Face mode of OCTA ensures precise localization of damages in certain retinal layers based on their axial location on SOCT cross-sectional scans and allows converting OCT images to other models of fundus visualization while retinal vessels serve as reference points [13].

Technical capabilities of the new methods enabled improvement of Gass classification for the En Face mode [9]:

– stage 0: foveolar profile remains unchanged on the cross-sectional OCT scan; central retinal thickness is normal; En Face mode reveals single diffuse foci of fibrosis on the retina's surface.

– stage 1: cross-sectional OCT scan shows increased central retinal thickness; the foveolar profile changes insignificantly; En Face mode reveals a "patch" with a small number of radial folds.

– stage 2: cross-sectional OCT scan shows increased central retinal thickness; the foveolar profile disappears; En Face mode reveals a solid "patch" with radial folds.

When examining a vitreoretinal interface with En Face OCTA, practitioners should pay particular attention to changes in the posterior hyaloid membrane (PHM) and ERM as the relate to the pathological process development. When focused on PHM, the retina's surface appears "cloudy" or "foggy". In cases of detachment of PHM or ERM, local separated parts appear as "retinal windows", which is a diagnostic indicator the surgeon should take into account when removing an ERM. Sometimes, the inner limiting membrane ruptures in places; then, En Face imaging reveals naked zones of the retinal nerve layer visualized as "craters" [9].

Best corrected visual acuity (BCVA) may not provide the data quality needed to correctly evaluate vision disorders related to various fundus pathologies. If that is the case, there is an alternative, computer-assisted microperimetry, a noninvasive method that allows estimating light sensitivity of the macular area and localization and stability of the point of gaze [15].

The analysis of pros and cons of the existing iERM diagnosis methods tells that getting a complete picture of morphofunctional changes associated with this pathology takes a multimodal approach. This study aimed to evaluate such a multimodal approach that includes ophthalmoscopy, multispectral examination, microperimetry, SOCT and En Face OCTA as they are applied in combination to diagnose stages of iERM.

METHODS

46 patients (46 eyes) took part in the study; they had iERM at varying stages. The inclusion criteria were pseudophakia and early cataract. The average age of participants was 65.3 ± 11.2 years (18 women and 27 men). The exclusion criteria were: mature cataract, glaucoma, diabetic retinopathy, fundus dystrophy regardless of its genesis, occlusion of retinal vessels, registered eye trauma, chronic and acute inflammatory eyeball diseases.

The standard ophthalmologic examination was complemented with SOCT, multispectral examination (MultiColor mode, various filters), microperimetry and En Face OCTA. The following instrumentation and devices were used: Spectralis HRA-OST, Spectralis OCT-2 module, frequency of 85,000 Hz with TruTrack Active Eye Tracking (Heidelberg Engineering, Germany); MAIA version 2.4.0 (CenterVue, Italy). SSDA algorithm was applied to OCTA (Angio Retina mode). The scanning was performed in the macular zone, gaze fixed centrally, size of the macular cube — 10 × 10°, number of scans — 512, distance of 6 μm (Macular Map mode). If the patient's gaze wandered, scanning was repeated until the images obtained were free from artifacts caused by eye movement.

SOCT data allowed evaluation of the degree of structural disturbances in vitreoretinal interface, overall central retinal thickness and foveolar profile. En Face OCTA images were used to assess frontal profile of the vitreoretinal interface, including internal limiting membrane and ERM within the macular zone.

Microperimetry allowed assessing photosensitivity of retina's central zone. The examination was performed in the

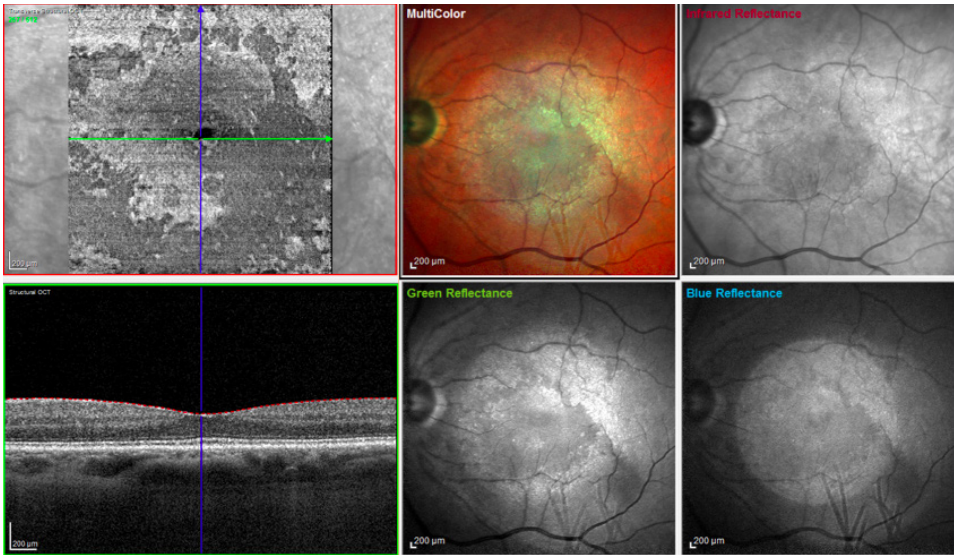

Fig. 1. Example of multimodal diagnostics results, iERM, stage 0. Membrane area and boundaries are almost invisible, no wrinkles/folds on the retina, no traction component, sporadical diffuse fibrosis foci

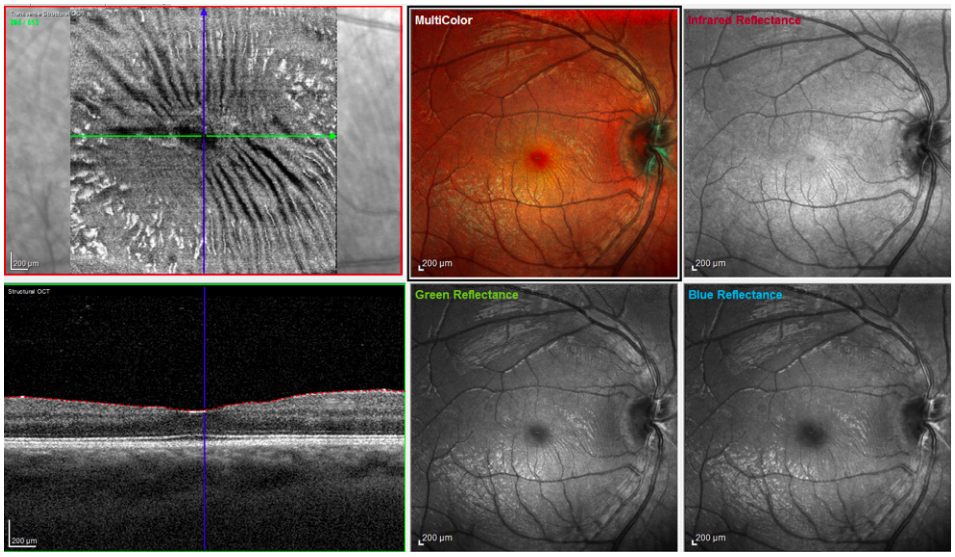

Fig. 2. Example of multimodal diagnostics results, iERM, stage 1. Membrane area and boundaries are well defined, sporadical wrinkles/folds on the retina, unpronounced traction, visible star patterns on the retina and patches

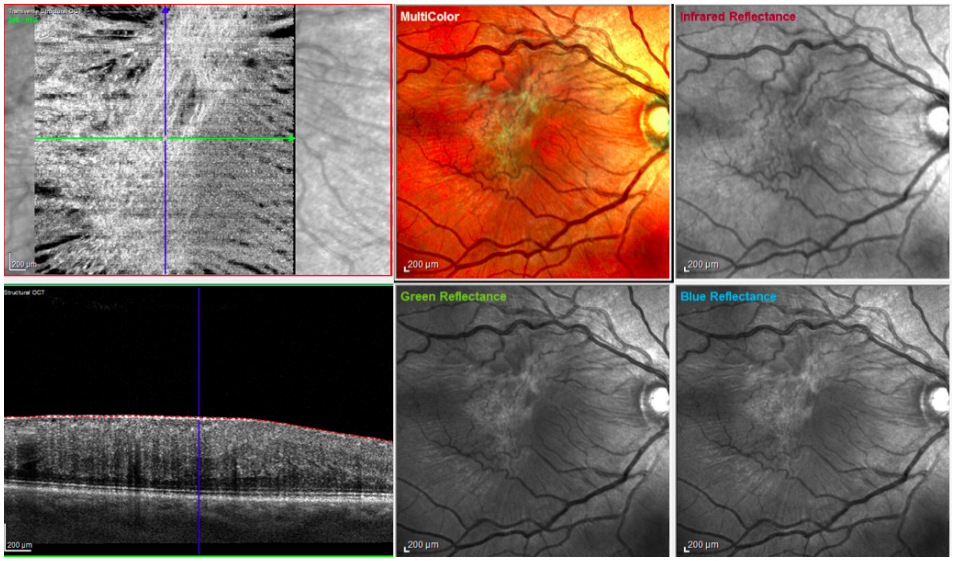

Fig. 3. Example of multimodal diagnostics results, iERM, stage 2. Membrane area and boundaries are clear, pronounced wrinkles/folds on the retina, pronounced traction, increased retinal thickness

macular zone, gaze fixed centrally, Expert exam mode [method 4–2], 10° grid (37 points). The results of the examination were superimposed on a picture of fundus shot with a built-in fundus camera. In case the patient registered more than 30 % of dots in the area of the blind spot, the test was deemed unreliable and was repeated to obtain reliable data.

RESULTS

Based on the results of the multimodal examination, the Gass classification and its modified version, we divided the patients into 3 groups: 15 patients (15 eyes) in the first group, all with initial manifestations of epiretinal fibrosis (stage 0); 19 patients (19 eyes) in the second group, with changes to vitreomacular interface more pronounced (stage 1); 12 patients (12 eyes) to the third group, with marked changes in the macular zone (stage 2).

Multispectral (MultiColor) examination of the first group showed weak yellow-green foci from ERM surface, localized in the center zone; the membrane's total area and boundaries could not be differentiated, there were no folds nor traction (Fig. 1). SOCT showed no changes to the foveolar profile, and the central retinal thickness was normal (average of 276.8 ± 24.4 nm). En Face images revealed single diffuse foci of fibrosis on the surface of retina of all patients. The average photosensitivity of retina in the macular zone was 26.2 ± 1.4 dB.

Multispectral (MultiColor) examination of the second group showed pronounced yellow-green reflection from ERM surface, localized in the center and helping to see the membrane's total area and boundaries; there also were some folds and weak traction (Fig. 2). SOCT showed minor changes to the foveolar profile (smoothening) and increased retinal thickness to the average of 303.1 ± 42.2 nm. En Face OCTA visualized "retinal stellata" and "patches" with individual radial folds in all patients. The average photosensitivity of retina in the macular zone was 25.1 ± 2.4 dB.

Multispectral (MultiColor) examination of the third group showed clearly pronounced yellow-green reflection, ERM area and boundaries clearly visible; traction was well-expressed and folds diffused (Fig. 3). COCT revealed gross structural changes to the retina, lack of foveolar profile, increase of the central retinal thickness to the average of 427.0 ± 85.4 nm. En Face images showed diffuse retinal folds in the central zone, all patients. The average photosensitivity of retina in the macular zone was 22.2 ± 2.2 dB. In 2 cases out of 12, a partial ERM detachment was observed: En Face OCTA showed "retinal windows". Thus, we were able to draw up the vitreoretinal interface topography scheme while taking into account the zones where ERM was farthest from the retina. Further on, we developed a map of vitreoretinal interface that allowed planning treatment and surgical removal of ERM with minimal trauma to the retina.

DISCUSSION

For many years, the "golden standard" in diagnosing epiretinal fibrosis was ophthalmoscopy and SOCT. However, new, non-invasive confocal microscopy diagnostic methods gave ophthalmologists more details on the development of proliferation at various stages of the disease.

Multispectral examination in combination with SOCT and En Face OCT allows diagnosing and assessing changes to iERM at different stages. What is more, the data describe not just cross sections but entire areas of the membrane. En Face OCT provides a deeper insight into the morphofunctional changes in the vitreoretinal interface. This method is not a routine one yet, but it can find wide use in diagnosing fundus pathologies.

The type and extent of symptoms a patient develops largely depend on the thickness of the pathological membrane, on retina deformation it causes, on its location and the presence/absence of macular edema or macular rupture. In the absence of complications, visual acuity can remain at a sufficiently high level, therefore BCVA does not allow correct assessment of proliferation dynamics. Here, microperimetry can help, since this method ensures control over the threshold of central retina photosensitivity and localization and stability of the point of gaze.

Combination of the "golden standard" and new, non-invasive diagnostic technology is a promising approach to uncovering iERM, drafting treatment tactics and observing dynamics.

CONCLUSIONS

Based on our own practical experience and the data obtained through the study, we can state that each of the diagnostic methods used separately does not yield a complete picture of morphofunctional changes peculiar to epiretinal fibrosis. Multimodal approach opens new opportunities before for the practitioner and provides more detailed data on the structure of vitreoretinal interface in iERM at various stages of its development; allows assessing dynamics of pathology not only by cross-sectional spectral OCT scans but also by area imaging enabled by MultiColor or En Face OCTA methods; helps determining the effect proliferation produces on the quality of vision using the microperimetry data. Such information allows better planning of treatment tactics, including scope and nature of surgical intervention.

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