THE EFFICIENCY OF PROBIOTIC BACTERIA IN COMBINED VACCINATION AGAINST HEPATITIS B IN EXPERIMENTAL STUDIES

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The aim of the work – to elucidate the efficacy of three times immunizations of BALB/c mice with recombinant Engerix-B vaccine, as well as its combinations with lactobacilli and bifidobacteria probiotic strains for hepatitis B prevention.

Materials and methods. The recombinant Engerix-B (ENGEXIR B) vaccine from GlaxoSmithKline Biologicals S.A. (Belgium) was used in the work. It was administered to mice subcutaneously along the spine once a day, three times with an interval of 10 days. The formation of antibodies to the HBsAg subtypes ad and ay in serum blood was studied by enzyme-linked immunosorbent assay.

Results and discussion. It was found that after immunization of mice with Engerix-B vaccine, specific antibodies to the surface antigen of viral hepatitis B (HBsAg) appeared from the 10th day after second vaccination. Application of the vaccine in combination with Lactobacillus casei IMV B-7279 caused increasing of specific antibodies concentrations only after three times immunizations, but it was higher than in other groups of animals. Combination of the vaccine with L. casei IMV B-7279 – L. acidophilus IMV B-7279 – L. delbrueckii subsp. bulgaricus IMV B-7279 or L. casei IMV B-7279 – Bifidobacterium animalis VKL – B. animalis VKB probiotic compositions was less effective: specific antibodies appeared after single-dose immunization, but the humoral immune response was very weak. In comparative studies of the antibodies to HBsAg formation of the virus subtypes ad and ay it has been established that application of Engerix-B vaccine for HBV prevention increases immune response to HBV subtype ay. The most significant difference was observed in case of using the vaccine in combination with L. casei IMV B-7280, after a 3-time immunization.

Conclusions. The use of Engerix-B vaccine in conjunction with L. casei IMV B-7280 increases the effectiveness of hepatitis B vaccination in animals, as evidenced by a significant increase in antibody titres after the third immunization.

Key words: hepatitis B, vaccination, lactobacilli, bifidobacteria, enzyme-linked immunosorbent assay.

Viral hepatitis B is an anthropogenic viral infection characterized by immunologically mediated hepatocyte lesion that occurs in various clinical forms. The causative agent of infection is DNA-genomic Hepatitis B virus (HBV) of the genus Orthohepadnavirus of the family Hepadnaviridae. The high contagiosity of the HBV, which in comparison exceeds the infectious dose of human immunodeficiency virus (HIV) in 100 times, and the variety of ways of its transmission (parenteral, domestic, vertical – mother-to-child transmission or transmission during labour and delivery) are the cause of the widespread prevalence of the hepatitis B among the population. According to the World Health Organization (WHO), the number of people infected with the HBV in the world exceeds 2.1 billion people; 1 million of them die every year. In people who had undergone hepatitis B, the disease often turns into a chronic form with a high risk of liver cirrhosis developing. The number of people infected with hepatitis B virus in Ukraine is about 20 thousands people [1]. However, it cannot be ruled out the increase in this quantity in recent years that is likely due to the increase in the volume of blood transfusions in the provision of assistance to victims of military operations in eastern Ukraine.

In carrying out measures to reduce the spread of hepatitis B, the main role is played by active specific immunization – vaccination for the prevention of hepatitis B. The surface antigen of viral hepatitis B of recombinant vaccines is obtained by application of recombinant DNA technology and adsorbed on aluminum hydroxide, are widely used. So, the surface antigen of the hepatitis B virus, derived from microbial cells, is purified by a series of physicochemical methods. In this case it is transforming into spherical particles with a diameter of 20 nm, containing non-glycosylated polypeptides and a lipid matrix consisting mainly of phospholipids [2]. Studies have shown that these
particles have properties that are typical for natural HBsAg. Their introduction into the body causes the formation of specific HBs-antibodies, which in the titer 10 IU/ml prevent the development of hepatitis B infection [3].

After immunization with these vaccines, immune memory is preserved for at least 12 years [4]. However, deterioration of socio-economic and environmental conditions leads to an increase in the number of people with various immunodeficiencies, in which the effectiveness of vaccination is reduced. It should also be noted that the majority of vaccinated children are children of the first year of life, when the formation of the immune system has not been completed, which affects the formation of a protective humoral response after vaccination. There is also a need to develop more effective vaccination methods that can provide adequate protection in reduced schedules. In connection with this, it is especially important to search for new effective vaccination schemes for the prophylaxis of viral hepatitis B, as well as the use of combination vaccines blending the target antigen with adjuvants, such as, for example, probiotic strains of lactobacilli or bifidobacteria [5, 6].

Probiotic strains of bacteria that have antagonistic, anti-inflammatory and immunomodulating effects are used both for prevention and treatment of many infectious diseases [6], and for increasing the effectiveness of vaccination [7]. It was shown that under the influence of some probiotics, the effectiveness of treatment of patients with hepatitis was increased [8], as well as immunogenicity of vaccines against hepatitis B [5] or C [9]. It should be noted that in the case of combined use of the vaccine and probiotics, the effectiveness of vaccination depended on the age of people, the antigen, the type of immune response to antibodies, and also the strain of bacteria. Therefore, the results of clinical studies concerning the use of probiotics in vaccination are promising and require further confirmation.

The purpose of our work was to determine the effectiveness of vaccination for the prophylaxis of viral hepatitis B when combined use of the Engerix-B vaccine with probiotic strains of lactobacilli and bifidobacteria.

Materials and methods

Experimental animals. The studies were performed using BALB/c mice (males) weighing 25-26 g (12-13 weeks). The animals were kept under standard vivarium conditions, in plastic cells in a separate room at a constant air temperature (20-25) °C. They received full-blown mixed fodder and had free access to the water. The maintenance and handling of experimental animals were carried out in accordance with the norms established by the Law of Ukraine No. 3447-IV «On the Protection of Animals against Cruel Treatment» and «European Convention for the Protection of the Rights of Vertebrate

Animals used for Experimental and Scientific Purposes from 09.20.1985» (Strasbourg, 1986) [10].

Probiotic strains of bacteria. Lactobacillus acidophilus IMV B-7279, L .casei IMV B-7280, L. delbrueckii subsp. bulgaricus IMV B-7281, Bifidobacterium animalis VKL and B. animalis VKB probiotic strains, obtained from the intestines of clinically healthy people, were used in the work. L. acidophilus IMV B-7279, L. casei IMV B-7280 and L. delbrueckii subsp. bulgaricus IMV B-7281 strains are deposited in the Depository of microorganisms of the D.K. Zabolotnyi Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine.

Prior to each experiment, the viability of the lactobacilli and bifidobacteria cultures was determined by a conventional method by seeding on Man-Rogosa-Sharpie (MRS) and MRS media with 0.5 % cysteine, respectively.

Vaccine against hepatitis B. The recombinant Engerix-B (ENERGIX B) vaccine from GlaxoSmithKline Biologicals S.A. (Belgium) with a concentration of HBsAg of 20 μg/ml was used in the work. In animal studies, it was diluted with phosphate buffered saline (PBS, pH 7.4) to 1.25 μg/ml.

Animal vaccination scheme. 500 μl of Engerix-B vaccine was administered to mice subcutaneously along the spine once a day, three times with an interval of 10 days. Simultaneously, the animals received an oral suspension of lyophilized probiotic bacteria diluted in phosphate buffered saline at a dose of 1x10⁸/mouse once a day for 7 days. In studies, L. casei IMV B-7280 was used in monoculture, and also in probiotic compositions of L. acidophilus IMV B-7279 – L. casei IMV B-7280 – L. delbrueckii subsp. bulgaricus IMV B-7281 (in the 1: 1: 1 ratio) and L. casei IMV B-7280 – B. animalis VKL – B. animalis VKB (in the 1: 1: 1 ratio).

In the experiments, four groups of animals were formed with 15 individuals each: 1) vaccinated mice; 2) vaccinated mice that received L. casei IMV B-7280; 3) vaccinated mice that received L. casei IMV B-7279 – L. acidophilus IMV B-7279 – L. delbrueckii subsp. bulgaricus IMV B-7281 composition; 4) vaccinated mice that received L. casei IMV B-7279 – B. animalis VKL – B. animalis VKB composition.

The peripheral blood sampling for the study was performed from the tail vein 10 days after each immunization. Blood serum was obtained by a conventional method and stored until analysis at minus 20 °C. Blood serum of 5 mice in each group was pooled and examined for antibodies to HBsAg after the 1st, 2nd and 3rd vaccinations.

Enzyme-linked immuno sorbent assay (ELISA) of serum. For the ELISA test, Medi Sorp LW (Nunc) 96-well polystyrene plates were sensitized with purified by chromatography viral HBsAg (Capricorn) on separate strips with ad and ay subtypes at a concentration of 0.5 μg/ml at 100 μl/well in carbonate-bicarbonate buffer (pH 9.2). Sorption was carried out at 4 °C for 20 hours. To block non-specific binding sites,
the plate was treated with a buffer solution of cow's milk extract (pH 7.2), which was introduced into the wells of the immunosorbent by 100 μl for 30 minutes, after which the plate was dried under vacuum and used for ELISA.

During the reaction, 70 μl of a PBS solution containing 0.05 % Tween 20, non-specific reaction blockers and preservatives, and 30 μl/well of test sera were added to the wells of the immunosorbent. The plate was incubated at 37 °C for 60 minutes and washed four times with PBS with 0.05 % Tween 20 to remove antibodies that did not bind to the immunosorbent. The resulting immune complexes were detected by goat anti-mouse polyclonal antibodies labeled with horseradish peroxidase, which were added at 100 μl/well in the optimal titer. After incubating the plates at 37 °C for 30 min, their washing was carried out as above, 100 μl/well of the TMB/substrate solution was applied to the reaction and incubated at (18-20) °C in the dark for 30 minutes. The reaction was stopped with 0.5 M H2SO4, which was added in the amount 100 μl/well. The analysis result was taken with a spectrophotometer in two wave modes at 450/620 nm. The result of the study of each test sample was calculated by the formula:

\[ \text{OD/ODc,} \]

where OD – optical density of the solution in the well when analyzing the test sample;

ODc – optical density in the well when analyzing the control (intact mice).

Results and discussion

When immunizing BALB/c mice with the Engerix-B vaccine, specific antibodies begin to appear 10 days after the second vaccination (Table 1). When this vaccine was administered in combination with one strain of lactobacilli – L. casei IMV B-7280 – antibodies to HBsAg were detected in significant titers only after three times immunization, but their concentration was significantly higher than in all the studied groups of mice. Compared to animals immunized with vaccine alone, the concentration of specific antibodies at the same period of observation was 1.4-1.6 times higher in mice receiving the vaccine and L. casei IMV B-7280. If the vaccination was carried out in combination with probiotic compositions – L. acidophilus IMV B-7279 – L. casei IMV B-7280 – L. delbrueckii subsp. bulgaricus IMV B-7281 or L. casei IMV B-7280 – B. animalis VKL – B. animalis VKB, then specific antibodies appeared after single-dose immunization, but the humoral immune response was significantly weaker.

In Table 1 also shown the dynamics of the detection of antibodies to the surface antigen of the HBV of ad and ay subtypes. It was found that with the use of Engerix-B vaccine for the prevention of hepatitis B, the immune response to the subtype ay prevails. Most of all, this difference is noted when using the vaccine in combination with L. casei IMV B-7280 after the third immunization.

Table 1

<table>
<thead>
<tr>
<th>No. in order</th>
<th>Composition of the combined vaccine</th>
<th>Blood serum sampling for analysis (days)</th>
<th>Anti-HBsAg antibodies to HBV subtypes</th>
<th>ELISA results (ODsample/ODcontrol*)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10 days after the 1st vaccination</td>
<td>10 days after the 2nd vaccination</td>
<td>10 days after the 3rd vaccination</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ad</td>
<td>ay</td>
<td>ad</td>
</tr>
<tr>
<td>1</td>
<td>Vaccine</td>
<td>0.5</td>
<td>0.6</td>
<td>1.4</td>
</tr>
<tr>
<td>2</td>
<td>Vaccine + L. casei IMV B-7280</td>
<td>0.7</td>
<td>0.7</td>
<td>0.8</td>
</tr>
<tr>
<td>3</td>
<td>Vaccine + L. casei IMV B-7280 + L. acidophilus IMV B-7279 + L. delbrueckii subsp. bulgaricus IMV B-7281</td>
<td>0.9</td>
<td>1.0</td>
<td>0.7</td>
</tr>
<tr>
<td>4</td>
<td>Vaccine + L. casei IMV B-7280 + B. animalis VKL + B. animalis VKB</td>
<td>1.0</td>
<td>0.9</td>
<td>0.6</td>
</tr>
</tbody>
</table>

* – The result is positive if the value of ODsample/ODcontrol is greater than 1.0; The result is negative if the value of ODsample/ODcontrol is less than 1.0
Based on the data we obtained, it can be argued that *L. casei IMV B-7280* can be an effective stimulator for the Engerix-B vaccine, since the concentration of specific antibodies after three times immunization was greatest in mice receiving the vaccine and this probiotic strain. Obviously, the use of *L. casei IMV B-7280* and the Engerix-B vaccine provides a more complete immune response to the antigen. Such effect of lactobacilli can be mediated both through their participation in the energy metabolism and the synthesis of biologically active substances, which plays a decisive role in the development of the immune response and direct immunomodulating effects at the local and systemic levels [6].

It is known that for the prevention of hepatitis B vaccines are required, under the influence of which both humoral and cellular links of immunity are strengthened, when the Th1/Th2 immune response is balanced towards the development of Th1-type cells and activation of cytotoxic T-lymphocytes. Aluminum hydroxide, which is currently widely used as an adjuvant in hepatitis B vaccines, effectively stimulates Th2-type immune response, but does not affect the Th1-mediated immune response [11]. At the same time, it is known that some probiotic bacteria can stimulate the production of interleukins-2, -12, as well as interferon-γ, promoting the development of Th1-type immune response [6].

Therefore, the introduction of such probiotic bacteria into the hepatitis B vaccination scheme is of great practical interest. In particular, it has been shown that the combined use of probiotics and vaccine against hepatitis B significantly increased the production of specific antibodies in children of the first year of life compared to children vaccinated only with vaccine [5]. Increasing the effectiveness of hepatitis B vaccination by stimulating intestinal bifidobacteria and minimizing the symptoms of dysbiosis, as established in young children, also supports the joint use of probiotics and vaccines [12].

The studied strains of probiotic bacteria *L. casei IMV B-7280, L. acidophilus IMV B-7279, L. delbrueckii subsp. bulgaricus IMV B-7281, B. animalis VKL and B. animalis VKB* in monoculture or in various compositions can activate phagocytic system cells, change the indices of cellular immunity and balance the production of Th1 and Th2-type cytokines [13]. Our results showed that the use of Engerix-B vaccine in conjunction with probiotic compositions – *L. acidophilus IMV B-7279 – L. casei IMV B-7280 – L. delbrueckii subsp. bulgaricus IMV B-7281 or L. casei IMV B-7280 – *B. animalis VKL – B. animalis VKB* – was less effective than combination of vaccine with only *L. casei IMV B-7280*. This is probably due to the fact that *L. casei IMV B-7280* is more efficient inducer of Th1-type cytokines than other probiotic bacteria we have been studying [13]. Apparently, the enhancement of the formation of specific antibodies in the case of Engerix-B vaccine and *L. casei IMV B-7280* introduction is due to the influence of *L. casei IMV B-7280* on the production of Th1 and Th2-type cytokines, but additional studies are needed to confirm this.

**Conclusions**

Thus, the use of Engerix-B vaccine in conjunction with *L. casei IMV B-7280* increases the effectiveness of hepatitis B vaccination, as evidenced by a significant increase in antibody titres after the third immunization. However, further research is needed to understand the mechanisms of the effects of probiotic bacteria on the balance of Th1- and Th2-type immune response when combined with the hepatitis B vaccine.

**Література**


References


ЕФЕКТИВНІСТЬ ПРОБІОТИЧНИХ БАКТЕРІЙ ПРИ КОМБІНОВАНІЙ ВАКЦІНАЦІЇ ПРОТИ ГЕПАТИТУ В В ЕКСПЕРИМЕНТІ

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Мета роботи – визначення ефективності трирації имунізації миші BALB/c рекомбінантною вакциною Енджеерикс-В, а також її поєднання з пробіотичними штамами лактобацилл і бифідобактерій для профілактики зепатиту В.

Матеріали і методи. У роботі використовували рекомбінантну вакцину Engerix-B (INGERIX В) ви- робництва GlaxoSmithKline Biologicals S.A. (Бельгія), яку вводили мишам підкірно по хребту один раз на добу, тричі з інтервалом в 10 діб. Формування HBsAg-антитіл до підтипів ad і ay у сироватці крові визначали за допомогою імуноензимного методу.

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Результати та обговорення. Встановлено, що після імунізації мишей вакциною Енджерикс-В специфічні антитіла до поверхневого антигену вірусного гепатиту В (HBsAg) починали виявлятися через 10 діб після другої вакцинації. При використанні вакцин в комбінації з Lactobacillus casei IMB B-7279 специфічні антитіла визначались в значних титрах тільки після триразової імунізації, адже їхня концентрація була вищою, ніж в інших групах тварин. Комбінація вакцин з композиціями L. casei IMB B-7279 – L. acidophilus IMB B-7279 – L. delbrueckii subsp. bulgaricus IMB B-7281 або L. casei IMB B-7279 – Bifidobacterium animalis VKL – B. animalis VKB була менш ефективною: специфічні антитіла з'являлися вже після одноразової імунізації, але гуморальна імунна відповідь була дуже слабкою.

У порівняльних дослідженнях динаміки утворення антитіл до HBsAg субтипів ad та ay встановлено, що при введенні вакцини Енджерикс-В превалює імунна відповідь до субтипу ay. Найбільше цю різницю виявлено при використанні вакцин в поєднанні з L. casei IMB B-7280 після третьої імунізації.

Висновки. Використання вакцини Енджерикс-В у поєднанні з L. casei IMB B-7280 підвищувало ефективність вакцинації проти гепатиту В у тварин, що підтверджувалося суттєвим підвищенням титру антитіл після третньої імунізації.

Ключові слова: гепатит В, вакцинація, лактобацилли, біфідобактерії, імуноферментний аналіз.

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