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Immunization with Recombinant Pneumolysin Induces the Production of Antibodies and Protects Mice in a Model of Systemic Infection Caused by *Streptococcus pneumoniae*

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Immunogenic and protective activity of recombinant pneumolysin was studied in experiments on male BALB/c mice. The mice were immunized intraperitoneally with recombinant pneumolysin sorbed on $Al(OH)_3$ (200 μg per mouse). In 2 weeks after immunization, the isotypes of antibodies to recombinant pneumolysin in the serum of immunized mice were determined by ELISA. The animals were infected with *Streptococcus pneumoniae* serotype 3. Immunization with recombinant pneumolysin induced the production of anti-pneumolysin antibodies, mainly of IgG1 subisotype. On day 21 after intraperitoneal infection with *S. pneumoniae* serotype 3 in a dose of 10^6 microbial cells, the survival rate of animals immunized with recombinant pneumolysin in a dose of 25 μg/mouse was 67% *vs.* 0% in the control ($p<0.001$). Recombinant pneumolysin could be considered as a promising protective antigen for inclusion in the serotype-independent vaccine against *S. pneumoniae*.

Key Words: *Streptococcus pneumoniae; pneumolysin; recombinant pneumolysin; antibodies; protective activity*

Streptococcus pneumoniae is the causative agent of otitis media, pneumonia, meningitis, and sepsis. Vaccines based on capsular polysaccharide antigens (polysaccharide Pneumo-23 and conjugated Prevenar and Synflorix) are used to prevent pneumococcal disease. Introduction of the conjugate vaccine for children into the national immunization schedules reduces the overall incidence of invasive pneumococcal infections decreases. However, the results of immunization programs for adults >65 years of age are not so unambiguous, though changes in the ratio of *S. pneumoniae* were observed. In particular, it was shown that serotypes of the polysaccharide vaccine not duplicated in the conjugated vaccine predominate in this age group [5], which is a pressing issue due to the increase in the life expectancy of the population on a global scale.

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Another problem is pneumococcal carriage that ranges from 27 to 65% in children and less than 10% in adult population [7]. Pneumococcal vaccination is correlated with a higher risk of pneumococcal colonization of the nasopharynx and increased antibiotic resistance, irrespective of the type of vaccine (conjugated or polysaccharide) and immunization schedule of the particular country [8].

In general, serotype replacement counterbalances the positive effect of reducing the incidence of disease caused by vaccine-type *S. pneumoniae* [3].

In view of these peculiarities and limitations of capsular antigen vaccines, serotype-independent vaccines are being developed, including recombinant vaccines based on conservative pneumococcal proteins. One of these proteins, pneumolysin, is a pore-forming toxin and the most important protein among the pneumococcal virulence factors [2,4]. Pneumolysin in high concentrations forms pores in the membranes of eukaryotic cells, thereby inducing their cytolysis and

promoting inflammation. It also plays an important role in colonization of the upper respiratory tract and dissemination [2].

The aim of our work was to study the immunogenicity and protective activity of recombinant pneumolysin (rPly).

MATERIALS AND METHODS

The strain of *S. pneumoniae* serotype 3 was obtained from the Collection of the Common Use Center of I. I. Mechnikov Research Institute for Vaccines and Sera. *S. pneumoniae* was cultured for 18-24 h on a solid nutrient medium (blood agar) at 37°C and 5% CO_2 in a CO_2 thermostat.

Male BALB/c mice (body weight 16-18 g, age 6-7 weeks) were obtained from Andreevka nursery.

For immunization, rPly with a molecular weight of 53 kDa was used. The preparation contained bacterial endotoxins in a concentration of 120 U/ml. The content of bacterial endotoxins was determined by the LAL test (*Limulus polyphemus* amoebocyte lysate) using Endosafe LAL reagent (Charles River).

For ELISA, rPly in phosphate buffer (2.5 μg/ml) was sorbed on a 96-well plate for 2 h at 37°C and then, antibody titers to rPly were determined as described elsewhere [6].

For evaluation of immunogenicity and protective activity, rPly was initially sorbed on $Al(OH)_3$ (Sigma) in 0.9 % NaCl for 12 h at 4°C (200 μg $Al(OH)_3$ per mouse).

In a preliminary experiment, the spectrum of antibodies to rPly was analyzed. To this end, the mice were twice intraperitoneally immunized with rPly in a dose of 25 μg per mouse. The blood was collected, and isotypes IgM and IgG and subisotypes IgG1, IgG2a, IgG2b, IgG3 were determined in pooled serum using commercial secondary peroxidase-labeled anti-mouse antibodies (Thermo Scientific). In the main experiment, antibody production was assessed by analysis of individual serum samples (the blood was taken from the caudal vein).

For the lethal challenge, the mice were intraperitoneally immunized with rPly in a dose of 25 μg (3 injections with a 14-day interval). For modeling generalized *S. pneumoniae* infection, mice of the control (n=10) and experimental (n=9) groups were injected intraperitoneally with *S. pneumoniae* serotype 3 in a dose of 10^6 microbial cells in 2 weeks after the last immunization. Observation was carried out for 21 days.

Statistical processing of the data was carried out using Microsoft Excel and Statistica 10 (StatSoft, Inc.). Mice survival (%) analysis was also evaluated using a log-rank test [1]. The differences were significant at $p<0.05$.

RESULTS

Analysis of pooled mouse serum after 2-fold immunization revealed a significant increase in anti-rPly antibodies of the IgG1 subisotype in comparison with the control group ($p<0.05$), while no increase in the titers of IgG, IgG2a, IgG2b, IgG3 to rPly was found. In further analysis, only the dynamics of the titer of IgG1 subisotype antibodies was evaluated in immunized mice.

Analysis of individual antibody titers in mice immunized with rPly in 2 weeks after the second and third immunizations revealed a significant increase in antibody titer in comparison with the control group (maximum titer 1:6400, $p<0.05$; and maximum titer 1:51,200, $p<0.01$, respectively). Antibody titers after the third immunization significantly increased in comparison with those after the second immunization ($p<0.001$).

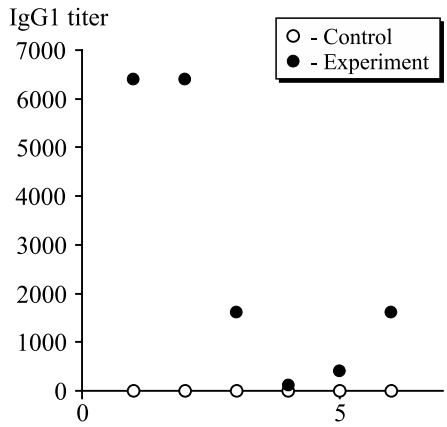

Fig. 1. Individual titers of antibodies in male BALB/c mice after the second immunization. Abscissa: numbers of individual blood samples from mice of the control and experimental groups. Here and in Fig. 2: each point represents individual titer of anti-pneumolysin IgG1 antibodies.

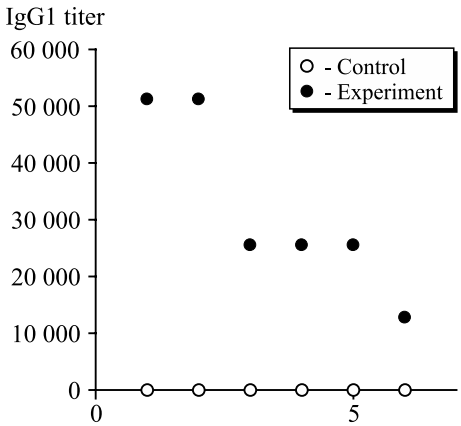

Fig. 2. Individual titers of antibodies in male BALB/c mice after the third immunization.

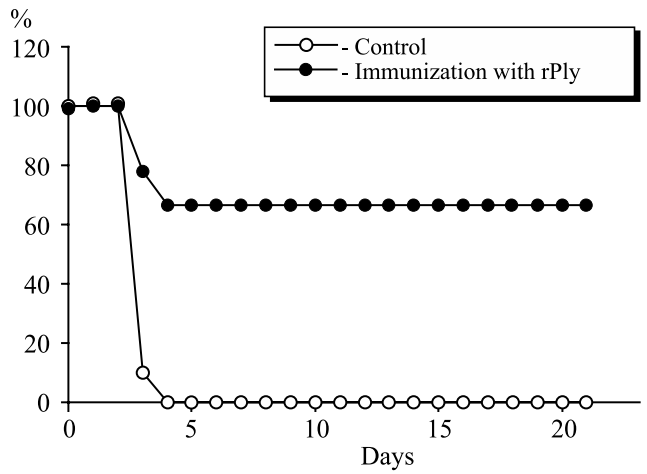

Fig. 3. Survival of male BALB/c mice in the control and experimental groups after intraperitoneal challenge with *S. pneumoniae* serotype 3 in a dose of 10^6 microbial cells.

The survival rate in rPly-immunized mice on day 21 after intraperitoneal challenge with strain *S. pneumoniae* serotype 3 was 66.7% *vs.* 0% in the control (all mice died on day 4; $p<0.001$).

Our results confirmed the promise of using rPly as the component of serotype-independent pneumococcal vaccines.

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