

# Immune Response in Mice Immunized with Live Cold-Adapted Influenza Vaccine in Combination with Chitosan-Based Adjuvants

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**Abstract**—An influence of intranasal combined injection of live cold-adapted influenza vaccine with chitosan derivatives as adjuvants on the subpopulation structure of mononuclear leukocytes of mouse spleen which reflects the orientation of the immune response was studied. It is found that the inclusion of chitosan preparations promotes activation of cellular-level of immune response.

**Keywords**—Immunophenotype, chitosan, cold-adapted vaccine, intranasal injection.

## I. INTRODUCTION

**I**NFLUENZA viruses, adenoviruses and rhinoviruses are common causative agents of acute viral infections of the upper respiratory tract. The most efficient prevention for influenza is the vaccination. Along with inactivated influenza vaccines, live cold-adapted (CA) influenza vaccines are used both in Russia and in United States. In contrast to inactivated influenza vaccine ones, live vaccines not only induce the formation of serum IgG, but also activate the local immune response and provide a degree of protection against drift variants of influenza virus. However efficient, current live influenza vaccines do not provide reliable protection in elderly. To increase the immunogenicity of influenza vaccines, a number of adjuvants were suggested and tested, including MF-59, aluminum hydroxide, polyoxidoonium etc.

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Natural chitosan-cationic polysaccharides obtained by deacetylation of chitin, a component of the shell of crustaceans, insects and fungi [3]–[10] have been used to increase the immunogenicity of inactivated influenza vaccines previously [1]. The mechanisms for the effect of the chitosan at the individual components of an immune response to inactivated vaccines are intensively studied. However, the effects of chitosan combined with live CA vaccine have not been described yet beside general observation that chitosan increases the immunogenicity and protective efficacy of live CA influenza vaccine [2]. This observation points that chitosane may help to augment immune response to influenza vaccination in elderly population.

Here we describe individual components of the immune response in mice immunized with live CA influenza vaccine in combination with chitosan derivatives delivered intranasally.

## II. MATERIALS AND METHODS

### A. Chitosan Preparations

Two preparations of chitosan, a 1% solution of chitosan glutamate (300 kD, deacetylation degree was 85%) and 1% suspension of micro/nanoparticles of chitosan sulfate, were tested. One percent solution of chitosan glutamate in 0.2 M glutamate buffer (pH 6.2) was added to equal volume of the vaccine to the final concentration of 0.5%. Micro/nanoparticles of chitosan sulfate were obtained by the following procedure. Carbamide peroxide was added to 1.2% aqueous solution of chitosan acetate sterilized by autoclaving to final concentration of up to 3% and stored for 36 hours at room temperature in tightly closed container, followed by an addition of 0.5 M sodium sulfate final concentration of 0.05 mol/L. The solution was vigorously stirred and left at room temperature for 30-60 min before the precipitate was formed. Resulting microparticles were washed with 0.05 M sodium sulfate solution five times by centrifugation (5 min at 3000 rpm); the pH of obtained suspension of micro/nanoparticles was 4.0-4.5. The resultant preparation was dried in a vacuum desiccator. 1% suspension of micro/nanoparticles was prepared by adding 0.3 volume of 0.2 M phosphate buffer pH 7.2, and 0.05 volume of 3 M NaCl to a suspension. Working solution pH was 6.4. The solution was added to the vaccine in equal volume to reach 0.5% concentration of

micro/nanoparticles. The pH of the resultant vaccine solution was 6.2 after its admixture with 0.2 M of the phosphate buffer.

### B. Influenza Strains

Cold-attenuated live influenza vaccine A/Krasnodar/101/35/59 (H2N2) was obtained by prolonged passage in 10-day-old developing chick embryos in suboptimal conditions (patent № 2354995 of Russian Federation). Acute respiratory infections in mice were induced by virulent epidemic strain A/Krasnodar/101/59 (H2N2).

### C. Study Design

Experiments were performed on male CBA mice weighing 10-12 g divided in 5 groups. The mice were placed under light ether anesthesia and immunized with different doses of CA strain with or without chitosan by introducing 25  $\mu$ l of the vaccine solution into each nostril. Immunization was repeated 20 days after the first CA vaccine administration. Study design included three arms: CA strain vaccination with 1% solution of chitosan glutamate adjuvant; CA strain vaccination with 1% suspension of chitosan sulfate adjuvant; CA strain vaccination in glutamate buffer solution (control). In all three cases the final mixture contained 106.0 of 50% end-point embryo infective dose of virus in 0.05 ml of vaccine solution. There were also two adjuvant control groups that received only the derivatives of chitosan with no CA strain vaccine, and no-vaccine no-adjuvant glutamate buffer control.

### D. Flow Cytometry

Flow cytometry analysis was performed using monoclonal antibodies (MAb) (Caltag Laboratories, USA) against cellular antigens and FACS Calibur (Becton Dickinson, USA). Cells were washed two times with cold phosphate saline buffer (FSB)/1% fetal calf serum (FCS) and stained with FITC- and PE-labeled antibodies according to manufacturer's instructions. Mononuclear leukocyte preparation from the mouse spleens were used for quantitation of T and B cell subpopulations using CD3, CD4, CD8; NK; CD25, SD19,  $\gamma\delta$ TCR, CD5 and MHC proteins of the II class. For the gating of the cell population, a combination of direct and side light scatter and cell size were used. At least 5000 cells were used for an analysis using the software package WINMDI 2.8.

### E. Cytokine Production

Splenocytes of immunized mice were pre-incubated for 24 h in growth medium RPMI 1640 or RPMI 1640 and phytohemagglutinin (5 mg/ml), then spontaneous and induced cytokine production was analyzed in supernatants. The cytokine level was determined using flow cytometer FACS Calibur (Becton Dickinson, USA) with test-system FlowCytomix Mouse Th1/Th2 10 plex with the application of beads sensitized by monoclonal antibodies to cytokines (GM-CSF, IFN- $\gamma$ , IL-1 $\beta$ , IL-2, IL-4, IL-5, IL-6, IL-10, IL-17, and TNF- $\alpha$ ) (Bender MedSystems, Austria).

## III. RESULTS

Immunophenotyping of the mice immunized with live CA influenza vaccine in combination with chitosan derivatives revealed the moderate increase in the number of lymphocytes displaying CD16 (NK), CD3/CD16 (NKT), CD71 and CD21 biomarkers.

The intranasal immunization of mice with live CA influenza vaccine A/Krasnodar/101/35/59 was accompanied by the decrease in the number of T cells with markers CD3 ( $61 \pm 5.2$  vs.  $35 \pm 2.0$ ,  $p < 0.01$ ) on the 7<sup>th</sup> day after the first immunization, this trend continued at the 1<sup>st</sup> and 7<sup>th</sup> day after the second immunization. The number of CD4-positive T cells also decreased two fold ( $36 \pm 2.1$  vs.  $17 \pm 1.5$ ,  $p < 0.01$ ) during the period of observations. Additionally, an inhibition of B1-, CD8-positive T-lymphocytes and MHC II positive cells was noted. The number of NK-cells increased by threefold ( $11 \pm 0.9$  vs.  $38 \pm 3.3$ ,  $p < 0.01$ ), and while the number of immune cells with  $\gamma\delta$ -TCR markers increased twofold ( $4 \pm 0.4$  vs.  $10 \pm 0.7$ ,  $p < 0.01$ ; and  $4 \pm 0.4$  vs.  $11.0 \pm 0.7$ ,  $p < 0.01$ ), as measured on the 1<sup>st</sup> and 7<sup>th</sup> day after immunization, respectively.

Intranasal immunization of mice with live CA influenza vaccine in combination with chitosan glutamate was accompanied by the following changes in subpopulations of mononuclear leukocytes: an increase in subpopulations of T cells expressing CD3 ( $61 \pm 5.2$  vs.  $47.0 \pm 3.5$ ,  $p < 0.01$ ;  $61 \pm 5.2$  vs.  $58.0 \pm 4.6$ ,  $p > 0.05$  as measured on the 1<sup>st</sup> and 7<sup>th</sup> day after the 2<sup>nd</sup> immunization, respectively), CD8 ( $24.0 \pm 2.1$  vs.  $15.0 \pm 1.1$ ,  $p < 0.01$  on the 7<sup>th</sup> day after the 1<sup>st</sup> immunization) and CD16 ( $11.0 \pm 0.9$  vs.  $48.0 \pm 4.4$ ,  $p < 0.01$  times on the 7<sup>th</sup> day after the 1<sup>st</sup> immunization). B1 lymphocyte subpopulations also increased in their numbers as evident by CD5-positive cell increase on the 1<sup>st</sup> day after the 1<sup>st</sup> immunization ( $4.6 \pm 0.3$  vs.  $5.8 \pm 0.6$ ,  $p > 0.05$ ) and on the 7<sup>th</sup> day after the 2<sup>nd</sup> immunization ( $4.6 \pm 0.3$  vs.  $6.3 \pm 0.9$ ,  $p > 0.05$ ). With the micro/nanoparticles of chitosan sulfate as an adjuvant, live CA influenza vaccine immunizations resulted in a similar tendency: an increase of T-lymphocyte subpopulations with markers CD3, CD8, CD16 and CD4 and B1-lymphocytes subpopulations.

In case of intranasal immunization with cold-adapted strain A/Krasnodar/101/35/59 (H2N2) the moderate activation was observed for cytokines IFN- $\gamma$ , IL-1 $\beta$ , IL-2 and IL-10 (activity augmentation by 4.0-, 2.0-, 2.5-, and 3.7-fold on the 7<sup>th</sup> day after first immunization, respectively). We could detect only a very slight increase in the activity of GM-CSF, IL-4, IL-17, IL-5 (increase by 1.3-, 1.6-, 1.5-, and 1.3-fold, respectively). The TNF- $\alpha$  level was dramatically reduced in the first week after first immunization. However, it should be noted that after the second intranasal immunization by CA strain there was observed marked elevation of most cytokines under study: INF- $\gamma$ , TNF- $\alpha$ , GM-CSF, IL-4, IL-2, IL-5, and IL-6 (the increase by 40-, 63-, 18-, 10.3-, 5.0-, 11.7-, 3.3-, and 3.2-fold, respectively).

Intranasal administration of live cold-adapted influenza vaccine to mice when this vaccine was combined with

chitosan glutamate resulted in the elevation of most cytokines; however this level was moderate as compared with the level being observed under intranasal immunization with live influenza vaccine alone. The chitosan inclusion in live cold-adapted influenza vaccine resulted in activation of cytokines such as IFN- $\gamma$ , IL-1 $\beta$ , IL-17, IL-2, IL-6 (the increase by 8.6-, 3.2-, 6.2-, 3.1-, and 3.9-fold respectively on the 7<sup>th</sup> day after first immunization). GM-CSF, IL-4, IL-5 and IL-10 activation was insignificant after first immunization (the increase by 2.1-, 2.2-, 1.2-, and 2.5-fold, respectively). Second immunization provoked higher activation of IL-2 and IL-6, as well as TNF- $\alpha$  (the increase by 9.1-, 8.1-, and 11.4-fold, respectively).

The inclusion of micro/nanoparticles of chitosan sulfate suspension in live CA influenza vaccine was accompanied by synthesis inhibition of some cytokines after first immunization: IFN- $\gamma$ , TNF- $\alpha$ , IL-17, IL-5 (the synthesis decrease by 2.7-, 10.0-, 5.0-, and 2.1-fold, respectively) and moderate activation of other cytokines under study: GM-CSF, IL-1 $\beta$ , IL-2, IL-10 (the increase by 1.6-, 1.7-, 1.9-, and 3.6-fold). After the second immunization pronounced elevation of TNF- $\alpha$ , IL-6 and IL-10 was observed (the synthesis increase by 15.3-, 37.0-, and 18.0-fold, respectively), as well as moderate increase in other analyzed cytokines.

While analyzing the production of early cytokines *in vivo* following mice infection with epidemic strain of influenza virus A/Leningrad/134/57 (H2N2) and its cold-adapted variants it was noted that after the infection with cold-adapted viruses the TNF- $\alpha$ , IFN- $\gamma$ , IL-12 and IL-6 levels were significantly lower than those under the infection with epidemic strain [12]. Our experiments also demonstrated the decrease in TNF- $\alpha$ , IFN- $\gamma$ , and IL-6 synthesis in splenocyte culture from mice immunized with CA strain A/Krasnodar/101/35/59 (H2N2) as compared with the level of the synthesis of these cytokines in mice infected with epidemic strain A/Krasnodar/101/59 (H2N2). While studying the cytokine profile of splenocytes from mice immunized with live cold-adapted influenza vaccine there was observed insignificant elevation of IL-4 and IL-5 and expressed activation of IFN- $\gamma$  synthesis that could evidence for the development of Th1-type immune response.

#### IV. DISCUSSION

The of live influenza virus vaccines is an expensive and time consuming process. An addition of adjuvant may enhance the immune response to live attenuated influenza virus vaccines and reduce the amounts of vaccine needed for an efficient protection. Recently, alpha-C-galactosylceramide (alpha-C-GalCer), has been shown to have adjuvant activity for live attenuated vaccines [11]. Additionally, Markushin and coauthors showed that 1% solution of chitosan glutamate may also be used as an adjuvant for the intact CA donor-strain of attenuated A/Krasnodar/10/35/59 (H2N2), as it leads to increase both in serum IgG levels of intranasally immunized mice and in the protective effect [2]. The results of the present study indicate that an intranasal injection of epidemic strain of

influenza A/Krasnodar/101/59 (H2N2) into mice that causes viral pneumonia and intranasal injection of donor-strain of attenuation A/Krasnodar/101/35/59 (H2N2), the number of subpopulations of CD3, NK/CD3, CD5 (B1-lymphocytes) and CD4-positive cells decrease.

Immune down-regulation action of vaccine virus is possibly the reason of low efficiency of live CA influenza vaccine in the elderly. The inclusion of chitosan derivatives into live CA influenza vaccine leads to the noticeable increase of CD3, CD16, CD4, CD8, CD5, and CD21-positive cells. It should be noted with similar efficacy study chitosan derivatives.

It should be emphasized moderate lowering in the synthesis of most cytokines in mice immunized with live cold-adapted influenza vaccine combined with chitosan derivatives at a late observation as compared with the cytokine synthesis in mice immunized with chitosan-free CA live influenza vaccine. This fact could be considered as a manifestation of immune-correcting action of chitosan under the elevated reactivity of the immune response.

#### V. CONCLUSION

In addition to the previous results on increase of humoral immune responses the results of the present research indicate that the inclusion of chitosan derivatives into live CA influenza vaccine also helps to activate the cellular level of the immune system. Thus, the effectiveness of vaccination with live CA influenza vaccine can be increased with the use of chitosan derivatives as adjuvants including the prevention of ENT diseases of viral etiology.

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