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ORIGINAL ARTICLE

Chitosan as an adjuvant for parenterally administered inactivated influenza vaccines

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Abstract The addition of 0.5% of a chitosan derivative to inactivated influenza vaccines injected parenterally resulted in a four or six to tenfold increase in antibody titres after a single-dose or two-dose intramuscular immunization of mice, respectively, in comparison with antibody titres after immunization without chitosan. Chitosan-adjuvanted vaccines enhanced antibody titers against drift variants of A- and B-type human influenza viruses four to six times compared with the vaccines without chitosan. Inactivated avian influenza virus A/H5N2 admixed with chitosan, when administered to mice challenged afterwards with the same virus, showed higher immunogenicity and protective efficacy compared with the antigen without chitosan.

Introduction

Inactivated influenza vaccines administered parenterally have been successfully used for influenza prophylaxis for more than 50 years [2, 20]. However, the majority of the existing inactivated vaccines are less effective in the elderly [6, 14] and are unable to protect the vaccines from influenza virus drift variants [1, 3].

Aluminium salts were previously used as adjuvants in order to enhance the immunogenicity of inactivated influenza vaccines. However, the only adjuvant that has been used recently for influenza vaccines is MF-59. MF-59-adjuvanted vaccine has proved to be more immunogenic compared with a nonadjuvanted vaccine in the elderly. However, addition of MF-59 increased the reactogenicity of the vaccine [16, 17].

Some recent reports describe the cationic polysaccharide chitosan as an effective mucosa adhesive adjuvant for a number of intranasally administered inactivated viral vaccines, including influenza vaccine [9].

In this study, we investigated a chitosan derivative as an adjuvant for inactivated influenza vaccines administered parenterally. The results obtained confirm that this preparation significantly enhances the immunogenicity of inactivated influenza vaccines and induces the production of antibodies against influenza virus drift variants.

Materials and methods

Vaccine antigens

We used trivalent split vaccines Vaxigrip for epidemical seasons 2003/04 and 2005/06, MG subunit vaccines (Russia) for the 2005/06 season and subunit vaccine Influvac for the 2005/06 season. Purified and inactivated strain A/Mallard duck/Pennsylvania/1021/84(H5N2) was used in experiments with avian influenza virus. The latter was adapted to mice and kindly provided by Dr. Smirnov [19].

Viruses

We used influenza virus strain A/New Caledonia/20/99 as an A/H1N1 strain recommended by WHO for the 2003/04, 2004/05 and 2005/06 influenza seasons; strain A/Panama/2007/99 (analogous to A/Moscow/10/99 strain), recommended by

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WHO for the 2003/04 influenza season, strain A/Wyoming/3/2003 (analogous to A/Fujian/411/2002 strain), recommended by WHO for the 2004/05 influenza season, and strain A/New York/55/2004 (analogous to A/California/7/2003 strain), recommended by WHO for the 2005/06 influenza season, as A/H3N2 strains. Strain B/Beijing/243/97 (analogous to B/Hong Kong/330/2001), recommended by WHO for the 2003/04 influenza season and B/Jangsu/10/2003 strain (analogous to B/Shanghai/361/2002), recommended by WHO for the 2004/05 and 2005/06 influenza seasons, were used as influenza B strains. In addition, a mouse-adapted avian influenza virus, A/Mallard duck/Pennsylvania/1021/84(H5N2), was used.

Chitosan

We used a 1% solution of a chitosan derivative (deacetylated by 85%) in 0.2 M glutamate buffer.

Immunisation

Equal quantities of 1% chitosan in glutamate buffer solution or of glutamate buffer solution alone were added to inactivated influenza vaccines (split and subunit). Each experimental group consisted of four to six Balb/c mice (6–8 weeks old). Mice were vaccinated intramuscularly with 0.2 ml of a preparation containing 3 μg of each vaccine component and 0.5% chitosan or buffer. In the experiments with inactivated avian H5N2 influenza virus, the preparation contained 3 μg hemagglutinin in 0.2 ml and 0.5% chitosan or buffer. Mice were vaccinated once or twice intramuscularly. After primary vaccine administration, mice received a booster immunisation dose with the same composition of antigens on day 21. Mice were anesthetized with ether and bled on the twenty-first day after the first vaccination and 10 days after the second one. In experiments with the mouse-adapted inactivated avian influenza virus, mice were lightly anesthetized with ether and challenged intranasally with 50 μl of different LD_{50} doses of H5N2 virus on the twenty-first day after the first and on the tenth day after the second vaccination. The survival rates of these mice were observed for 10 days following vaccination.

Analysis of hemagglutination-inhibiting and neutralizing antibodies

Serum was heat-inactivated for 30 min at 56°C. Initial serum dilutions were 1:10, and then twofold dilutions of each sample were made. Serologic responses were measured according to standard techniques [10]. In the neutralization test, twofold dilutions of the initially diluted samples were prepared. Each twofold dilution (25 μl) was incubated with 100 EID_{50} of virus (25 μl) for 1 h at room temperature (24°C). Following incubation, two chick embryos were inoculated with each dilution of every sample and were incubated at 36°C for 48 h. The allantoic fluid was tested for hemagglutination with 0.5% chicken erythrocytes.

Statistical analysis

Three to four independent experiments were performed and the data were analyzed for statistical significance by Student test. Results with $P < 0.05$ were considered significant.

Results

Analysis of different chitosan concentrations

Chitosan in various concentrations (0.005, 0.05, 0.5 and 1%) or buffer solution alone were added to monovalent subunit vaccine MG (A/H3N2) containing 3 μg/0.2 ml of antigen. Mice were vaccinated two times intramuscularly. Analyses of antibody titres were performed with virus strains corresponding to those in the vaccines. Serum investigation showed that addition of 0.5% chitosan resulted in tenfold increase in hemagglutination-inhibiting and neutralizing antibody titres compared to control immunization without chitosan ($P < 0.01$). Lower chitosan concentrations induced a smaller increase in antibody titres. A concentration of 1% did not result in a further increase in titres (Fig. 1).

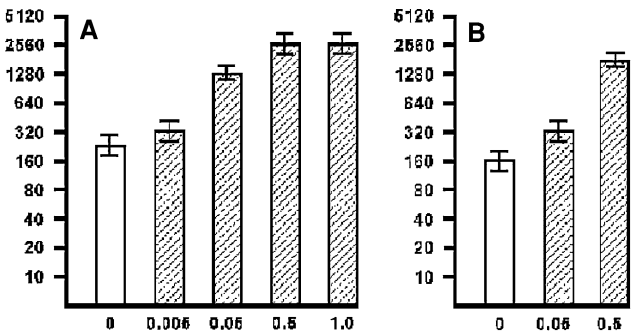

Fig. 1 Influence of different chitosan concentrations on the production of antibodies against A/H3N2 monovalent vaccine. Mice were immunized twice intramuscularly with the monovalent subunit vaccine MG containing an A/H3N2 influenza strain. Mice were bled on the tenth day after the second vaccination. Analysis of antibody titres was performed with the virus strain corresponding to the strain in the vaccine. **a** Titres of hemagglutination-inhibiting antibodies; **b** titres of neutralizing antibodies. *Open bar* vaccine without chitosan, *striped bar* vaccine with chitosan. *y*-Axis: serum dilutions, *x*-axis: chitosan concentration (%)

Influence of chitosan on production of antibodies against three serotypes of influenza viruses

In this experiment we used trivalent subunit vaccine Influvac (2005/06) as well as split vaccines MG (2005/06) and Vaxigrip (2004/05). Mice were vaccinated twice with 0.2 ml of antigen containing 3 μg of hemagglutinin of each viral serotype and 0.5% of chitosan. Antibody titres were evaluated with the viral strains corresponding to those in the vaccines. The mean results of six experiments are presented in Fig. 2. Addition of chitosan led to a six to tenfold increase in hemagglutination-inhibiting and neutralizing antibody titres for the A/H1N1 influenza virus serotype, eight to tenfold and six to eightfold increases for the A/H3N2 serotype, and six to eight or eightfold increase in titres for influenza type B viruses ($P < 0.01$). No significant differences could be found between split and subunit vaccines. We should point out that at the end of the experiment the weight of the mice vaccinated with and without chitosan did not vary.

Antibody titres did not increase in the experiment in which vaccine and chitosan solution were injected into different limbs of the mice (Fig. 2).

Antibody response after single-dose vaccination

Mice were vaccinated intramuscularly with 0.2 ml of split (Vaxigrip, 2005/06) or subunit (MG, 2005/06) vaccines. The preparation contained viral antigen (3 μg of each serosubtype) and 0.5% chitosan. Mice were bled on the twenty-first day after vaccination. In the study with vaccine without chitosan, mice were bled on the twenty-first day after the first or on the tenth day after the second vaccination. The results revealed that hemagglutination-inhibiting antibody titres after single-dose immunisation were four to six times (MG vaccine) or three to four times (Vaxigrip vaccine) greater than the titres after vaccination without chitosan ($P < 0.05$) (see Fig. 3). Moreover, antibody titres after single-dose vaccination with chitosan-adjuvanted vaccine exceeded those after two-dose vaccination with pure vaccine ($P < 0.05$).

Antibodies against influenza virus drift variants

Mice were vaccinated twice with Vaxigrip vaccines for the 2004/05 and 2005/06 influenza season. About 0.2 ml of the administered preparations contained 3 μg of viral antigens of the three viral subtypes and 0.5% chitosan. Control groups were vaccinated with a vaccine without chitosan. Hemagglutination-inhibiting and neutralizing antibody responses were evaluated with viral strains included in the above-mentioned vaccines as well as with the drift variants of those strains. Investigation of serum samples obtained from the mice vaccinated with Vaxigrip (2004/05 season) admixed with chitosan showed that the hemagglutination-inhibiting antibody titres against drift variants A/Panama/2007/99(H3N2) (recommended for the 2003/04 season) and A/New York/55/2004 (recommended for the 2005/06 season) were three times lower compared with antibody titres against the strain included in the vaccine (A/Wyoming/3/2003). In the same experiment, the titres of antibodies against influenza B drift variants B/Beijing/243/97 (recommended for the 2003/04 season) were four times lower compared with the antibody titres against the vaccine strain used for immunization (B/Jangsu/10/2003) ($P < 0.05$) (see Fig. 4). The titres of neutralizing antibodies against drift variants of A/H3N2 and B type influenza virus were three times lower than the titres of antibodies against the vaccine viruses that were used for vaccination ($P < 0.05$). Nevertheless, after the immunization of mice with a vaccine admixed with chitosan, antibody titres measured in HAI and neutralization tests were six times higher for A/H3N2 drift strains and four times higher for influenza B drift variants compared with the titres of antibodies against drift variants when a vaccine without chitosan was administered ($P < 0.01$ and $P < 0.05$, respectively).

In the experiments with Vaxigrip vaccine, recommended for the 2005/06 season, after vaccination without chitosan, titres of hemagglutination-inhibiting antibodies against drift variants A/Wyoming/3/2003 (recommended for the 2004/05 season) and against A/Panama/2007/99 (2003/04) were three and four times lower, respectively, compared with the A/H3N2 vaccine strain (A/New York/55/2004). Titres against influenza B drift variant virus B/Beijing/243/97 (2003/04 season) were three times lower than those against the vaccine influenza B strain (B/Jangsu/10/2003) ($P < 0.05$). In the neutralization test, antibody titres against A/H3N2 and influenza type B drift variants were three times lower than those obtained with the original vaccine virus ($P < 0.05$). However, sera of mice vaccinated with chitosan-containing vaccine revealed four to six and fourfold increases in hemagglutination-inhibiting antibody titres against A/H3N2 and type B influenza virus drift variants, respectively, compared with the vaccine administered without chitosan ($P < 0.05$). Neutralizing antibody titres against drift variants of A/H3N2 and type B influenza viruses were eight and four times higher, respectively, in comparison with murine sera obtained from mice vaccinated without chitosan ($P < 0.05$). The data obtained show that chitosan-adjuvanted inactivated influenza vaccines in comparison with vaccines without chitosan enhanced antibody titres, not only to the homologous strain but also to drift variants of influenza virus.

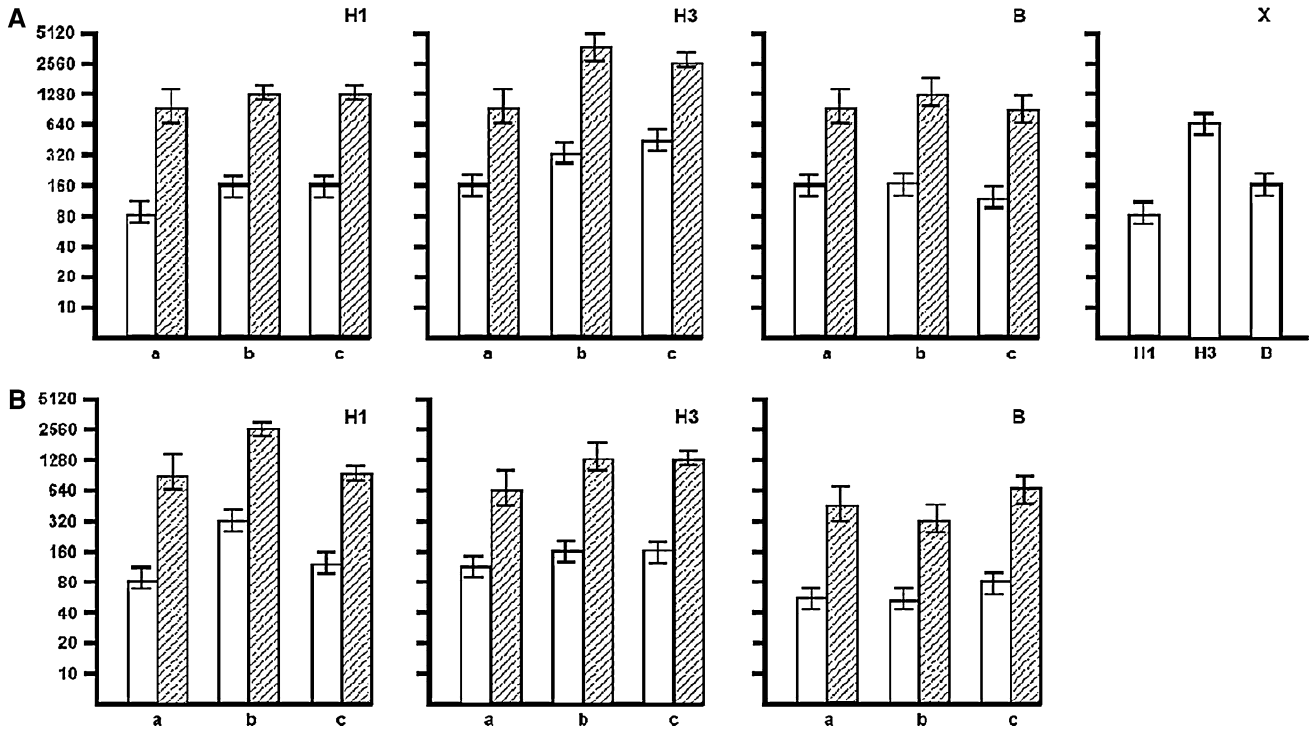

Fig. 2 Titres of antibodies against all three influenza virus serotypes after vaccination of mice with chitosan-containing inactivated vaccines. Mice were immunized twice with trivalent subunit vaccines MG 2005/06 and Influvac 2005/06 and split vaccine Vaxigrip 2004/05. Analysis of antibody titres was performed with virus strains corresponding to those contained in the vaccines. **a** Titres of hemagglutination-inhibiting antibodies; **b** titres of neutralizing antibodies, *H1*, *H3*, *B* serotypes of influenza viruses; *x* titres after separate injection of vaccine and chitosan into different limbs of the mice; *a* vaccine MG for the 2005/06 influenza season; *b* vaccine Vaxigrip for the 2004/05 season; *c* vaccine Influvac for the 2005/06 season; *open bar* vaccine without chitosan, *striped bar* vaccine with chitosan. *y*-Axis: serum dilutions. All of the differences observed in titres of antibodies induced by vaccines with and without chitosan were statistically significant ($P < 0.01$)

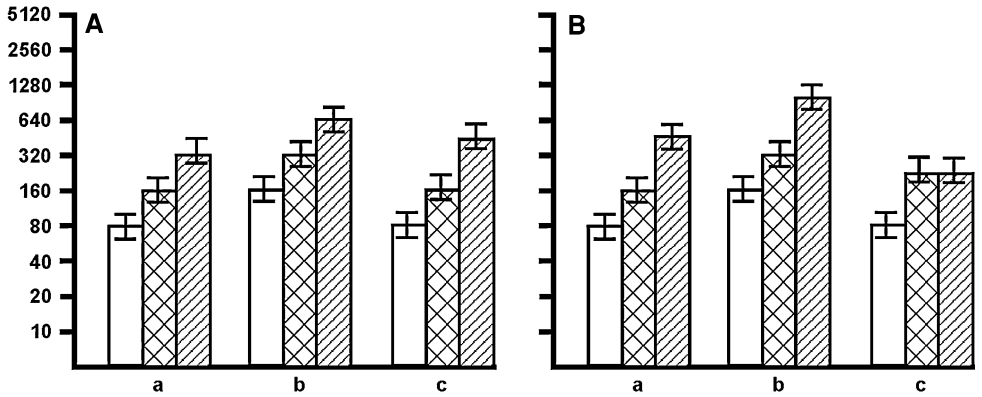

Fig. 3 Titres of hemagglutination-inhibiting antibodies after single and double vaccination of mice with chitosan-adjuvanted vaccines. Mice were immunized with subunit vaccine MG 2005/06 or split vaccine Vaxigrip 2004/05. **a** Vaccine MG 05/06; **b** vaccine Vaxigrip 04/05. *a* A/H1N1 strain; *b* A/H3N2 strain; *c* B strain. *Open bar* vaccine without chitosan, titres after the first vaccination. *Crobbed bar* vaccine without chitosan, titres after the second vaccination. *Striped bar* vaccine with chitosan, titres after the first vaccination. *y*-Axis: serum dilutions. All of the differences observed in the antibody titres induced by vaccines with and without chitosan were statistically significant ($P < 0.05-0.01$)

Experiments with avian influenza virus A/H5N2 (A/Mallard duck/Pennsylvania/10218/84)

Mice were vaccinated with purified and inactivated avian influenza virus by intramuscular injection of 0.2 ml of a preparation containing 3 μg of viral hemagglutinin and 0.5% chitosan. The data obtained show that after a single-dose vaccination with chitosan the antibody titre revealed by HAI test was 1:160, which is eight times higher than that following vaccination without chitosan (1:20). After

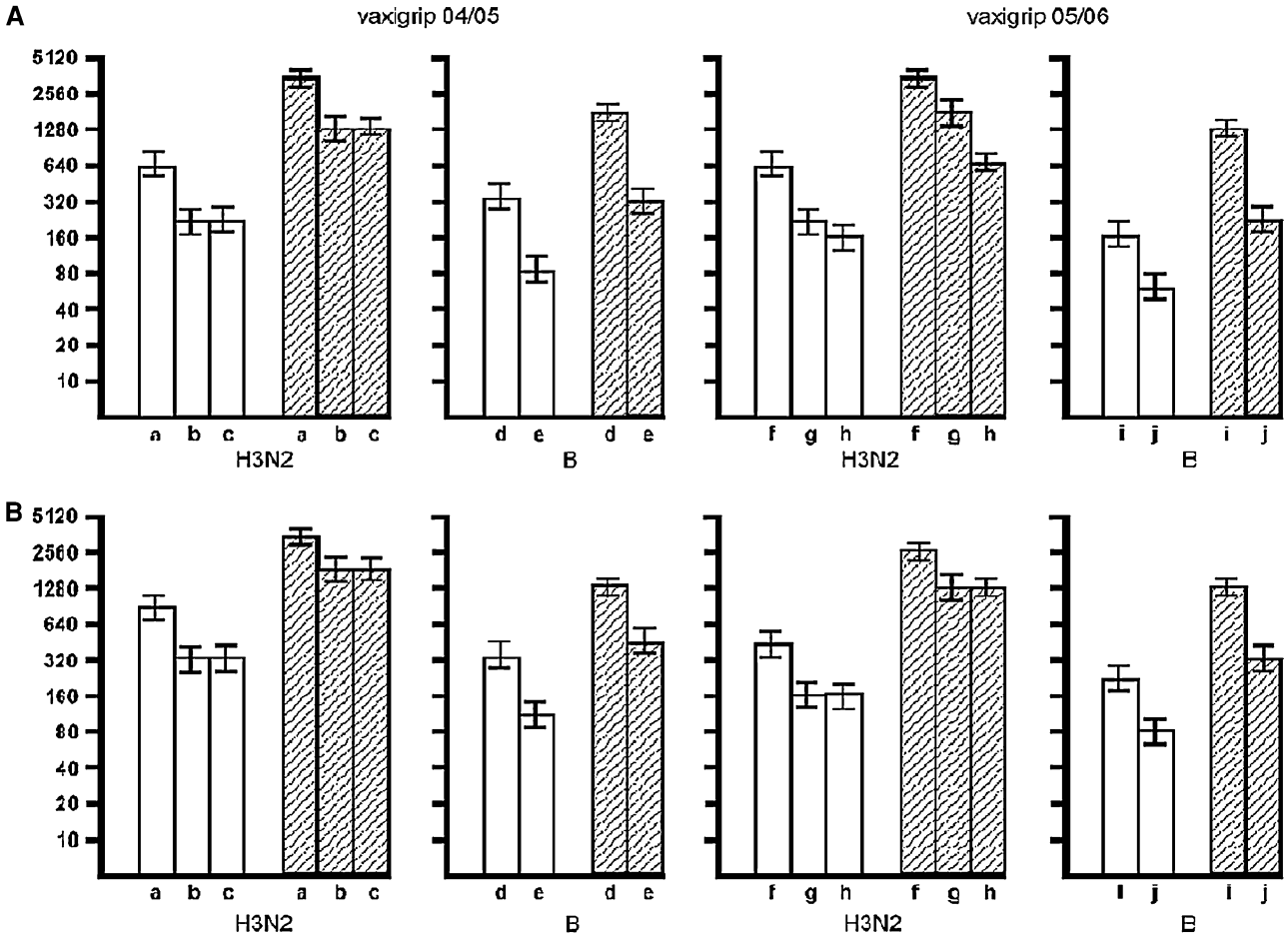

Fig. 4 Titres of antibodies to the drift variants of the influenza virus after vaccination with chitosan-containing inactivated vaccines. Mice were immunized twice with Vaxigrip vaccines for the 2004–05 and 2005–06 seasons. Analysis of antibody titres was performed with virus strains corresponding to those in the vaccines and with drift variants of A/H3N2 and B influenza viruses. **a** Titres of hemagglutination-inhibiting antibodies; **b** titres of neutralizing antibodies, *a* A/H3N2 strain, corresponding to the Vaxigrip vaccine strain for the 2004–05 season, *b* drift variant of the A/H3N2 strain of the influenza season 2004–05, recommended for season 2005–2006, *c* drift variant of the A/H3N2 strain of the influenza season 2004–05, recommended for season 2003–2004, *d* B strain, corresponding to the Vaxigrip vaccine strain for season 2004–05, *e* drift variation of B-type virus contained in the vaccine for season 2004–05, recommended for season 2003–04, *f* A/H3N2 strain, corresponding to the Vaxigrip vaccine strain for season 2005–06, *g* drift variant of A/H3N2 strain of the season 2005–06, recommended for season 2004–05, *h* drift variant of A/H3N2 strain contained in the vaccine of 2005–06 season, recommended for season 2003/04, *i* B strain, corresponding to the Vaxigrip vaccine strain for the 2005–06 season, *j* drift variant of B-type virus contained in the vaccine for the 2005–06 season, recommended for 2003–04 season; *open bar* vaccine without chitosan; *striped bar* vaccine with chitosan. *y*-Axis: serum dilutions. All of the differences observed between the titres of antibodies to the influenza virus drift variants after vaccination with chitosan and without it were statistically significant ($P < 0.05-0.01$)

two-dose vaccination the titre was 1:5,120, which is 16 times higher compared with the antibody response after vaccination without chitosan (1:320) ($P < 0.05$). The protective effectiveness of the single-dose and two-dose vaccination was evaluated. After vaccination, mice were challenged with the same mouse-adapted A/H5N2 strain (see Table 1), and survival rates were calculated. The level of protection after a single-dose vaccination without chitosan was 100, 100, 33 and 0% when challenged with 1 LD50, 10 LD50, 100 LD50 and 1,000 LD50, respectively. Administration of the virus adjuvanted with chitosan resulted in 100% protection when 1, 10 and 100 LD50 were used and in a 50% protection rate when challenged with 1,000 LD50. Two-dose vaccination resulted in 100, 100, 60 and 33% protection rates when infected with 1, 10, 100 and 1,000 LD50, respectively, when virus without chitosan was administered, and in complete (100%) protection when virus with chitosan was injected, even with the infection dose $1000LD_{50}$. The data show that chitosan-adjuvanted inactivated avian influenza virus enhances the immunogenicity and protective effectiveness of the inactivated virus ($P < 0.05$).

Table 1 Protection of mice vaccinated with inactivated avian influenza virus A/H5N2 with and without chitosan

Groups of mice	Challenge after the first vaccination infection dose (LD50)				Challenge after the second vaccination infection dose (LD50)			
	1	10	100	1,000	1	10	100	1,000
Vaccination with 0.5% chitosan	0/6[a]	0/6	0/6	3/6	0/6	0/6	0/6	0/6
Vaccination without chitosan	0/6	0/6	4/6	6/6	0/6	0/6	3/6	4/6
Nonvaccinated mice (control)	2/6	6/6	6/6	6/6	3/6	6/6	6/6	6/6

Mice were vaccinated once or twice intramuscularly with inactivated A/H5N2 influenza virus, containing 3 μg of hemagglutinin and 0.5% of chitosan or buffer in 0.2 ml. The second vaccination was performed on day 21 after the primary vaccination. Mice were infected intranasally with various doses of the same strain (mouse-adapted A/H5N2 influenza virus). Mice were challenged on day 21 after the first vaccination or 10 days after the second one. After the challenge mice were observed daily for 10 days

[a] The numerator represents the number of dead mice, and the denominator represents the number of infected mice

Discussion

Because of the threat of an influenza pandemic, there is growing interest in improving immunogenicity and protective effectiveness of the existing influenza vaccines. WHO noted that an increase in the quantity of antigens in a vaccine is nonrealistic because it would conflict with a rapid increase in the quantities of produced vaccines. Therefore, WHO recommends the investigation of new adjuvants capable of enhancing vaccine immunogenicity and effectiveness without increasing the antigen dose [15].

One of the first adjuvants used for inactivated influenza vaccines was aluminium salts. However, a number of studies did not reveal any significant advantages of vaccines with such an adjuvant when human vaccination is considered [18, 20]. The interest in this adjuvant has recently increased, especially in the case of the single-dose vaccination [7, 13]. At present, an MF-59-adjuvanted vaccine is being produced. It is characterized by high immunogenicity, especially in elderly subjects [16, 17].

Currently, mucosa adhesive adjuvants are the subject of great interest due to their ability to induce a local as well as a systemic antibody response against influenza when admixed with inactivated influenza vaccines and administered intranasally. Chitosan is an adjuvant from this group. Experiments performed on animals confirm that chitosan-adjuvanted intranasally administered vaccines induce immunity to influenza, whooping cough and diphtheria [9]. It should be noted, however, that when mass vaccination of people with an intranasal influenza vaccine adjuvanted with mutant *E. coli* toxin was performed [5] there were some complications, such as Bells palsy syndrome [12].

Our investigations were aimed at studying chitosan as an adjuvant for inactivated influenza vaccines administered parenterally. The data obtained show that chitosan significantly enhances the immunogenicity of the vaccines administered to mice intramuscularly. Our results revealed that the use of chitosan induces relatively high antibody titres even after a single-dose vaccination.

It is known that protection of inactivated influenza vaccines against human influenza virus drift variants is very weak [1, 3, 11]. In our study, antibody titres against influenza virus drift variants after parenteral vaccination with vaccines admixed with chitosan were much higher than after vaccination without chitosan. We have no data that chitosan acts to broaden the spectrum of antibody reactivity. But taking into account that chitosan-adjuvanted vaccine enhances the antibody titre to drift variants, it is possible to suggest that protection against influenza virus drift variants could be better with chitosan-adjuvanted vaccines than with vaccines without chitosan.

Recently, the possibility of a pandemic caused by avian influenza (e.g., with A/H5 virus) has been a subject of great anxiety. The results obtained in the present study with the mouse-adapted A/H5N2 duck influenza virus show that vaccination of mice with chitosan-adjuvanted inactivated virus enhances immunogenicity and protective effectiveness of the inactivated virus.

It should be noted that chitosan is widely used in the food industry and in medicine [4, 8].

The data obtained have allowed us to assume that the chitosan derivative investigated in the present study can become a promising adjuvant for parenterally administered inactivated influenza vaccines.

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