ASSESSMENT OF THE IMMUNOGENICITY AND PROTECTIVE EFFECTIVENESS OF REFLUVAC® IN MICE CHALLENGED WITH A PANDEMIC A/H1N1 INFLUENZA

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Abstract

This article describes the results of a pre-clinical study of immunogenicity and effectiveness of an inactivated pandemic vaccine (Refluvac®) on model mice. Mice received two 0.5 ml intraperitoneal inoculations with an interval of 14 days in three doses: containing 10.0, 5.0 and 2.5 µg HA (hemagglutinin) per animal. As a comparator preparation, the study used a semi-finished product (SP) vaccine diluted with phosphate buffered saline (PBS) to obtain HA concentrations of 5 µg and 10 µg. For a control group, the study used PBS as the negative control. We determined the vaccine's protective effectiveness level by analyzing its response in animals challenged with a pandemic A/California/7/09 (H1N1) pdm09 virus.

We assessed the immunogenicity of the vaccine by examining the mean geometric titre (GMT) of antibodies against the influenza virus as measured by hemagglutination-inhibition test (HAI). In the course of testing the GMT, we noted a dependence of the concentration of antibodies in serum on the vaccine's antigen load. The highest GMT was observed in the group of mice vaccinated with a HA load of 10.0 µg – it amounted to 278.6 (95% CI, 135.6 to 572.4). We established a high tolerability of the vaccine tested. Our study shows that Refluvac® yields a high degree of protectivity against influenza A/H1N1 and prevents clinical signs, death or accumulation of influenza virus in the organs of vaccinated animals. There were deaths and clinical signs including general depression, hypodynamia and anorexia in the negative control group. The results of our study were used for the clinical study of the first Kazakhstan-produced Refluvac® vaccine against pandemic A/H1N1 influenza virus.

Key words: Influenza A/H1N1, vaccine, safety, immunogenicity, mice.

Introduction

Influenza viruses can infect humans, swine and birds; and swine have long been considered a potential source of new influenza viruses capable of affecting people (Munster *et al.*, 2009). The emergence and circulation of new influenza virus type A/H1N1v, a triple reassortant, caused mass infections in 214 countries and made WHO declare the 6th phase of pandemic alert, meaning the start a pandemic, in 2009 (Zimmer & Burke, 2009).

The danger of A/H1N1 stems primarily from the lack of immunity to this virus type in most people and, hence, its ability to spread widely and cause severe conditions in those infected. Such a high potential of the virus is attributed to its mutability due to a segmented negative single-stranded mRNA (Webster *et al.*, 1992). The segmented genome serves as a basis for gene reassortion in mixed infections that can lead to new virus variants. It is considered that the influenza antigen shift most often takes place in swine,which can become infected with human and avian influenza simultaneously and thus produce new dangerous viruses (Webster, 1998). Human influenza genes give the new virus variant (reassortant) an ability to infect humans, while avian and swine influenza genes make the reassortant capable of causing severe conditions in humans by affecting rapidly both the upper and lower airways (Lipatov

et al., 2004). The lack of immunity to such viruses in humans facilitates a fast spread of the infection. Every year, Kazakhstan reports over 1 million cases of acute respiratory diseases and influenza, which at times take the form of epidemic outbreaks, occurring with varying intensity from October to January. In 2006 – 2007, the National Sanitary Epidemiological Service of Kazakhstan Ministry of Health isolated 19 strains of influenza type B and A (H1N1). Given the significant prevalence of influenza and the threat of it penetrating into Kazakhstan, prevention and effective control of the infection are impossible without nation-wide immunization compaigns. Seasonal influenza prevention campaigns immunize up to 500 thousand people, around 3.4% of the country's population, annually. Vaccination coverage varies from 6.8% to 10.2% among urban population and from 1.6% to 2.5% of the rural population. According to specialists, the influenza immunization coverage should be increased to 15 – 20% of the country's population with priority given to risk groups to create the necessary protective threshold (Hickling & D'Hondt, 2006).

No new vaccine is absolutely safe and not all of its risks can be identified before it becomes commercially available. The use of vaccines in clinical practice requires rigorous evidence of their safety and effectiveness. To secure such evidence, there are established procedures of research, the most important

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of them being the assessment of immunogenicity and preventive effectiveness in mouse models against a controlled and lethal infection with the principal virus strain in the vaccine tested. Therefore, the goal of this study was to examine the immunogenicity of a double inoculation with Refluvac® and assess the preventive effectiveness of the vaccine in mouse models challenged with A/H1N1 virus.

This study examines Refluvac®, an inactivated, adjuvant-based, pandemic vaccine for A/H1N1v influenza virus, developed jointly by Research Institute for Biological Safety Problems (RIBSP) of Kazakhstan and Influenza Research Institute of the Russian Federation to provide specific prevention of A/N1N1v. Refluvac® is an inactivated, whole-virion influenza monovaccine with an aluminun hydroxide adjuvant. The preparation was produced based on the NIBRG-121xp vaccine strain obtained from the National Institute of Biological Standards and Control (NIBSC, United Kingdom). The vaccine strain was produced through reverse genetic engineering and contains HA (hemagglutinin) and NA (neuraminidasa) protein genes of A/California/7/2009 (H1N1)v influenza virus and protein genes PA, PB1, PB2, NP, M and NS from the high-yield A/PR/8/34 influenza virus strain. The strain is recommended by WHO for making inactivated vaccines against AH1N1v. The vaccine was produced at the industrial facilities of RIBSP.

Materials and Methods
Vaccine

Samples of Refluvac® were prepared at RIBSP using NIBRG-121xp strain (code NIBSC: 09/166) produced by NIBSC (United Kingdom) by reverse genetic engineering from influenza viruses A/California/7/2009 (H1N1) pdm09 and A/PR/8/34(H1N1). We cultivated the vaccine virus in 10 – 11 days' old SPF embryos (Loman-Tirtsuht, Germany) for 72 hours at 37 °C. We then inactivated the viruses by incubating them with formaldehyde (Sigma, Germany) at 37 °C. We adsorbed the purified virus concentrate on aluminum hydroxide (Alhydrogel solution) using aluminum ions (Brenntag). We developed a monovalent pandemic vaccine using a technology for whole-virion candidate vaccines adsorbed on aluminum hydroxide. In scaling up this technology, we used new approaches to purifying virus-containic allantoic fluid, making it possible to obtain a viral concentrate with lower ovalbumin content. We assessed the vaccine's quality parameters in accordance with specifications provided by the manufacturer's Pharmacopeia. The vaccine was then handed over for an assessment of immunogenicity and effectiveness against influenza virus in an experiment involving a double vaccination of animals.

Animals

We used 18 – 22 g female BALB/C mice (n=180) to test the immunogenicity and preventive effectiveness of three experimental Refluvac® vaccine series. The experimental animals were grouped randomly. The randomization criteria used in the study was the absence of external signs of disease and the groups' homogeneity in terms of the animals' body weight (± 20%).

Assessment of immunogenicity and preventative effectiveness in mouse models challenged with influenza virus after double vaccination

Each vaccine preparation series was tested in three doses: 10 μg, 5 μg and 2.5 μg of hemagglutinin per animal. As a comparator agent we used a semi-finished Refluvac® vaccine product with no aluminum hydroxide or merthiolate containing 10 μg of HA per animal. We used PBS for negative control (pH=7.2) as the control group (n=15).

Animals were immunized twice by intraperitoneal introduction of 0.5 ml vaccine. The interval between the two immunizations equaled 14 days.

On the 14th day after the second vaccination, we collected blood samples to assess the immunogenicity by examining influenza antibodies using hemagglutinin inhibition test (HAI) (Anderson et al., 2012). Blood samples were taken from the tail vein (5 mice/group).

To test animal blood serum for assessing the vaccine's immunogenicity, we used influenza virus strain A/California/7/09 (H1N1) pdm09. We removed non-specific inhibitors from serum samples by treating them with a receptor-destroying enzyme from Vibrio cholerae (Denka Seiken Co. Ltd., Japan). We added eight hemagglutinating NIBRG-121xp viral units to serial dilutions of the tested serum samples in PBS and incubated this mixture at 37 °C for 30 minutes. We then added a 0.5% suspension of chicken red blood cells and performed sedimentation. We determined the antibody titre based on the highest serum dilution that inhibited viral hemagglutination. The detection limit of the HAI test was 10. We analyzed the significance of difference in GMT between the groups using the Turkey multiple comparison test with 95% CI.

We then assessed the preventative effectiveness of Refluvac® in model animals after two immunizations. To do this, we challenged mice with influenza A/California/7/09 (H1N1) pdm09 strain, which is capable of causing lethal influenza in mice (Bosch et al., 2010). Before challenging mice, we determined the LD50 of the epizootic wild strain. To do it, we made 10x dilutions of the virus in buffered saline solution from 10 – 1 to 10 – 9 and infected mice intranasally with 0.3 ml under light ether anaesthesia. We then observed the mice for clinical signs for 14 days. We calcuated the LD50 using the Reed and Muench

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method (Reed & Muench, 1938). We then challenged mice with the epizootic strain of avian influenza A/California/7/09 (H1N1) pdm09. We introduced the virus into anaesthesized animals intranasally, 30 ul suspension in a dose of $10^{5.5}$TID$_{50}$ (9.0 MLD$_{50}$) virus per animal. All tests involving pandemic virus were conducted in BSL-3 (Bio Safety Laboratory, level 3) environment. We observed the mice for 14 days for clinical signs, including daily body weight checks.

We also analyzed the challenged animals' body weight dynamics throughout the whole observation period and the necropsy of mice in the control group that died.

Statistical Analysis

We performed the statistical analysis of the whole experiment using GraphPad Prism Software, version 6.0 (GraphPad Software Inc., CA, USA) and StatSoft software, version 12. We analyzed the statistically significant dose-dependent differences between groups (weight, GMT) using the One-way analysis (Dunnett's test). We analyzed the survivability of challenged mice using the Logrank test.

Results and Discussion

Vaccine's immunogenicity in mice

Immunoprotectivity studies are a requirement for developing seasonal and pandemic influenza vaccines (Krammer *et al.*, 2014).

We planned this study in a way that we could supplement our previous research (Tabynov *et al.*, 2012) where we tested the vaccine's immunogenicity and effectiveness in ferrets. In that previous research we used vaccine samples with 3.75, 7.5 and 15.0 μg of viral hemagglutinin. The results, which are published in this article, show that a two-time vaccination for A/H1N1 provides protection against the infection. The influenza infection is similar in ferrets and humans

in terms of the symptoms, course of disease, viral circulation within the body and humoral immune response (Langlois, 2005; Gustin *et al.*, 2011; Belser, Katz, & Tumpey, 2011; Pearce *et al.*, 2012). According to literature, mice, along with ferrets, are often used in experiments on influenza vaccines' immunogenicity (Margine & Krammer, 2014; Thangavel & Bouvier, 2014; Scallan, Lindbloom, & Tucker, 2016).

In this study, we assessed the immunogenicity of Refluvac® in mice after a double vaccination. It should be noted that the GMT as measured in HAI test was dependent on HA concentration in the vaccine. The GMT in test animals were within the following ranges: 60.6 (95% CI, 22.73 to 161.8) for vaccines with a HA concentration of 2.5 μg/dose; 91.9 (95% CI, 20.0 to 160.0) for vaccines with a HA concentration of 5.0 μg/dose; 278.6 (95% CI, 135.6 to 572.4) for vaccines with a HA concentration of 10.0 μg/dose; while the concentration of antibodies in response to the semi-finished product of the vaccine tested with a HA load of 10.0 equaled 45.95 (95% CI, 22.36 to 94.4), (Fig. 1).

It indicates that all the tested vaccine samples provide a high level of immunogenicity (Fig. 1). There is a significant difference in GMT between mice inoculated with the tested vaccine samples having an HA concentration of 2.5 μg per dose and the control group inoculated with PBS (P=0.01). We also observed a significant difference between the group immunized with a 5.0 μg HA vaccine and the group inoculated with a 10.0 μg HA vaccine (P=0.001). Also, the group inoculated with a HA concentration of 10.0 μg had a significantly different GMT from the control group inoculated with PBS (P=0.0001). The difference in immunogenicity between test groups was insignificant. The study also showed the serum antibody level's dependence on the antigen load of the vaccine.
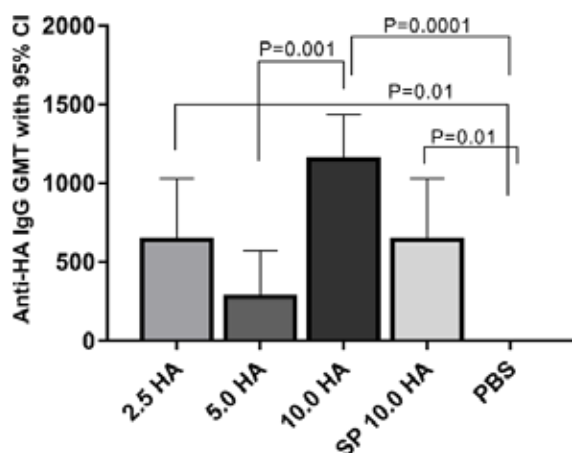

Figure 1. The results Refluvac® immunogenicity assessment in HAI depending on the HA antigen load of the vaccine tested (2.5 μg, 5.0 μg and 10.0 μg). Means are reported with standard errors (SEM).

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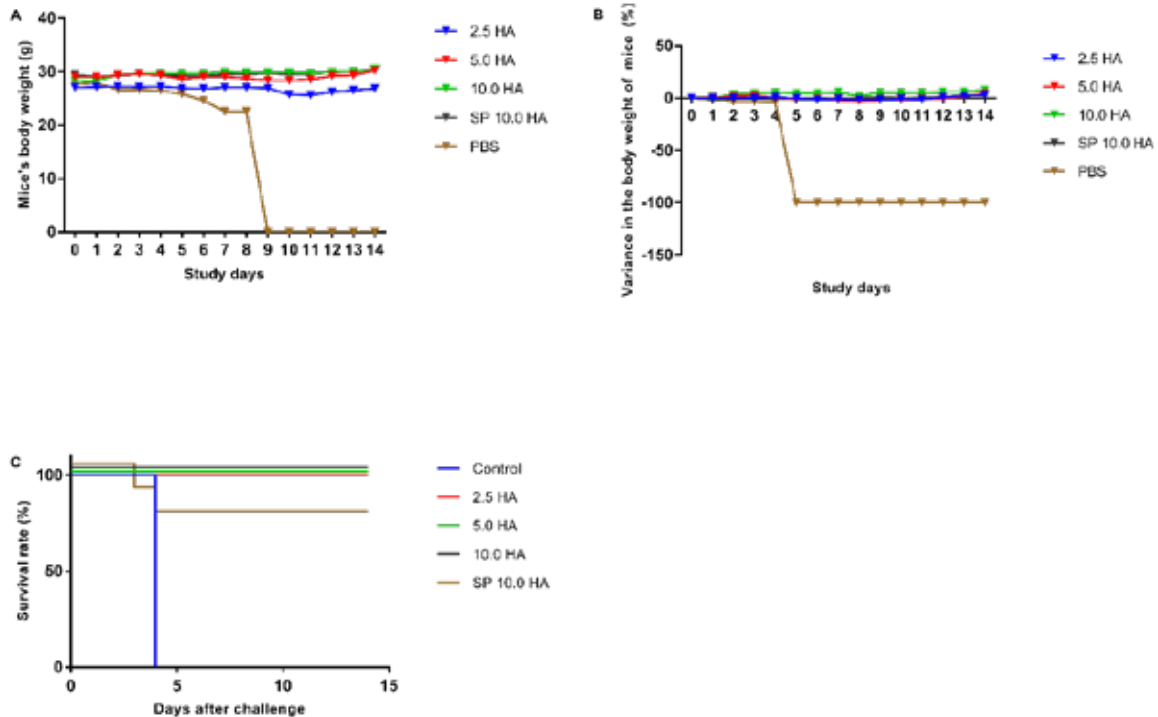PANDEMIC A/H1N1 INFLUENZA

Figure 2. Assessment of Refluvac® protective effectiveness. *(A) Body weight of experimental animals in grams (mean value). (B) Analysis of percentage variance in the body weight of experimental animals after challenge (using One-way analysis and Dunnett's test). (C) The survival rate of the challenged mice as observed during a 14-day period; comparison of the survival curves of all vaccinated groups and the control group (Logrank test). All test groups showed significant* difference to the control group (P<0.05).

The most important stage in this study was to assess the protective features of the inactivated vaccine depending on the adjuvant used (Hehme *et al.*, 2004). The study showed that (Fig. 2) all inactivated vaccine samples, whether adjuvant was used or not, provided a valid protection (P<0.05) of vaccinated mice from influenza A/H1N1. In our research, where mice were challenged with an epizootic virus, we observed that all tested doses of Refluvac® and its semi-finished product made from it provide a strong level of protection, prevent influenza clinical signs and deaths of vaccinated animals.

Weight loss was seen after the two immunizations in all groups. However, in spite of a slight loss at the beginning, by the end of observation all groups gained weight.

An analysis of body weight loss dynamics in challenged mice showed that mice vaccinated with Refluvac®, irrespective of the antigen load, developed a slight loss of weight during the observation period (14 days), but gained 2.7 g to 3.7 g by the end of it. Maximum weight gained was in mice inoculated with higher doses of the vaccine.

Similar dynamics was noted in mice vaccinated with the comparator agent; however, by the end of the experiment the weight gain in this group was insignificant and amounted to 0.8 g.

Mice in the control group, because of pronounced clinical signs including loss of appetite, started to lose body weight 4 days after challenge and showed the peak loss on the day 7 (-3.9 g).

The desired effect of vaccination is to elicit protective immune responses against infection with pathogenic agents (Jang & Seong, 2013; Music *et al.*, 2016). This experiment demonstrated that Refluvac® provides effective protection in challenged animals (Fig.2 C). The death rate in the control group was 100%. Before death, animals manifested signs of hypodynamia, dishevelled hair, tachypnea and lack of appetite. Mice in the negative control group began to die on the 4th day of the experiment. The same signs and deaths were observed with the group of mice immunized with the semi-finished Refluvac® vaccine (HA of 10 HA per animal), but the deaths in this case started on the sixth day of observation.

The necropsy of mice in the control group that died on the 4th to 8th day after being infected with A/California/7/09 (H1N1) pdm09 strain revealed signs of acute congestive hyperemia of lungs, subcutaneous tissues, liver, kidneys; acute enteritis, and petechial hemorrhages in the mucosa of the small bowel in some mice – all of which are typical of acute infection.

Our study of the immunoprotectivity of Refluvac®, an inactivated, adjuvant-based, pandemic vaccine for

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H1N1 influenza virus, introduced intraperitoneally in mice shows that the vaccine provides adequate immunogenicity and protection from A/H1N1 influenza virus.

Conclusions

Vaccine's immunogenicity demonstrates that Refluvac® yields strong immunogenicity in vaccinated mice inducing a high antibody titre against influenza virus A/H1N1, whose hemagglutinin features in the NIBRG-121x vaccine strain. The highest GMT results as measured by HAI test were in mice inoculated with the 10.0 µg HA vaccine; their titre as measured by HAI amounted to 278.6 (95% CI, 135.6 to 572.4). We also noted that the GMT is dependent on the dosage and presence of adjuvant in the preparation. The adjuvant used in the vaccines was aluminum hydroxide. Aluminum hydroxide enhances the vaccines' effectiveness to a large extent and allows for a maximum reduction of the antigen dose, thus reducing the vaccine's reactogenicity (ability to produce adverse reactions).

Our results demonstrate that Refluvac® is effective for A/H1N1 influenza virus and produces a specific immune response to A/H1N1 in mouse models. After two intraperitoneal vaccinations of mice, all test doses of Refluvac® (2.5 µg, 5.0 µg and 10.0 µg of HA per mouse) induced protective immunity. This was evidenced by a 100% survivability of animals after being challenged with A/California/7/09 (H1N1) pdm09 influenza virus and absence of external signs of disease or loss of body weight. The group of mice inoculated with Refluvac® semi-finished product with a HA dose of 10 µg/mouse also developed protective immunity but showed external signs of disease and had and insignificant number of animal deaths. The results of this study along with previous research helped us select the optimal HA dose ensuring a strong immunity and provide grounds to recommend this preparation for further clinical studies.

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