Seroconversion and immunogenicity evaluation of seasonal influenza vaccine in mice model

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Abstract Influenza virus is epidemiologically very important due to the antigenic variations occurring in it. In present study isolation and identification of influenza virus was attempted from Kasauli and adjoining areas. 71 clinical samples i.e. throat washings were processed and inoculated into the eggs for virus isolation. The isolated strains of H3N2, H1N1 and Influenza B were identified, purified and passaged in eggs. The harvested virus was inactivated by 0.03 % formalin and three formulations of crude, inactivated, whole virus monovalent vaccine for H3N2, H1N1 and influenza B as well as a trivalent vaccine was prepared. The immunogenicity study of the vaccine was performed in the mouse model by using varying dilutions of four types of vaccines for a period of 40 days. The impact of booster dose was also analyzed in the study. A significant seroconversion was observed in the mice sera verifying that the monovalent and trivalent lots of influenza vaccine were antigenically effective based on the serological tests.

Keywords Influenza vaccine · H3N2 · H1N1 · Type B · Mice immunogenicity · NISC · India

Introduction

Influenza term describes the ‘influence’ of factors of environment such as cold temperature on the occurrence of this epidemic respiratory disease [1, 2]. Influenza viruses are orthomyxo-viruses with a RNA which is single stranded and segmented. There are three types of influenza viruses A, B, and C. However, the subtypes of Influenza A are differentiated as H1N1, H2N2 and H3N2 on the basis of hemagglutinin (HA) and neuraminidase (NA) glycoproteins [7].

Influenza viruses are important respiratory pathogens causing of epidemics with high mortality. Influenza viruses are transmitted mainly by droplets and aerosols generated by infected people. Clinical symptoms associated with infection include high fever, headaches, cough, sore throat, runny nose and muscle ache that resolve within several days. Other associated complications of influenza infections include viral pneumonia, secondary bacterial pneumonia, airway inflammation [9, 10]. Nasopharyngeal samples are used for the isolation of the virus while using serum samples serological diagnosis can be carried out [3–5].

In India seasonal behavior of influenza illustrate outbreaks during the late autumn and winter months in temperate regions. The influenza activity is usually present throughout the year with a rise during the rainy season [6]. Annual seasonal influenza epidemics are of major public health concern as they are affecting 5–15 % of the global population with significant morbidity and mortality. The World Health Organization (WHO) reported an estimated 3–5 million cases of severe illness and 250,000–500,000 deaths worldwide due to seasonal epidemics [8].

The effective and widely used measure for the prevention of influenza infection is vaccination. WHO

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surveillance network keep regular monitoring of the circulating strains of seasonal influenza viruses. The most circulating strains of both influenza A and B are selected and the combined seasonal vaccine is prepared to be used in the patients.

Whole virus vaccines generally stimulate the development of circulating antibodies to the envelope proteins of the virus. It has repeatedly been pointed out that viral proteins are retained during inactivation process to generate optimal production of antibodies. This process is applicable to monovalent as well as trivalent vaccines.

Present study had been conducted at National Influenza Surveillance Centre (N.I.S.C) of Central Research Institute. The isolates had been detected, propagated and isolated in the specific pathogen free (SPF) embryonated eggs. The surveillance of the local influenza isolates are carried in association with local hospitals.

Materials and methods

Sample collection

The patients were identified in the local hospitals and dispensary on the basis of symptoms of influenza like illness like sneezing, cough, persistent fever, runny nose and the nasopharyngeal swabs were collected in Earle's balanced salt solution (EBSS). These samples were transferred in the ice pack boxes at 2–8 °C to the N.I.S.C. All the samples were centrifuged at 1200 rpm for 15 min in a refrigerated centrifuge and stored at –70 °C till further processing.

Virus isolation

Clinical sample was inoculated to 11 day embryonated eggs of white leghorn chicken by allantoic as well as amniotic route for virus propagation. Eggs were incubated at 37 °C for 2 days and then chilled at 4 °C overnight.

Allantoic and amniotic fluids were harvested and hemagglutination test was performed using fowl red blood cells. The sterile samples exhibiting the positive HA activity were included in the study. The unknown antigens were identified by performing hemagglutination inhibition test for titrating against known antiserum obtained from WHO.

Vaccine preparation

The virus isolates were concentrated by polyethylene glycol treatment. After the concentration the titre was determined and it was found to be on the rise. The virus isolates were inoculated in allantoic and amniotic cavity of embryonated hen's eggs. The eggs were incubated for next 48 h after which eggs were chilled overnight at 4 °C and then harvested next day. The allantoic fluid was syringe filtered. Virus harvests were collected aseptically for different isolates. For checking the sterility, the allantoic fluid was inoculated into fluid thioglycollate media and Soyabean casein digest media and incubated at 37 °C and room temperature for 14 days to check the sterility.

A small batch of vaccine was prepared by using three strains H3N2, H1N1 and influenza type B of influenza. The inactivation of virus particles was done with formaldehyde. It was found that the optimal inactivation of virus isolates was accomplished at a concentration of 0.03 % formalin, at 37 °C for 4 h. Crude viral vaccine for individual isolates was prepared and then the HA test was performed to check the HA titre. The three strains i.e. H3N2, H1N1 and influenza type B were pooled together to prepare a trivalent vaccine. The HA titre of the trivalent vaccine was also determined by HA test.

Immunogenicity in mice

The antigenicity of the monovalent and trivalent vaccine was evaluated by using mouse model. Four types of vaccines were used to immunize each of 30 Swiss albino mice were inoculated with different dilutions of the vaccines (0.5 ml) by intraperitoneal route. The inoculated mice were

Fig. 1 Antibody titre due to immunization with H3N2 monovalent vaccine. *The vaccine induced significant antibodies with single dose of vaccine after 21 days. **On booster dose the antibody level

showed a fall in titre and the fall remain constant for each dilution on 28th day. ***After 40 days there was a rise in antibody titre and this rise was almost same to the antibody titre at 21st day

Fig. 2 Antibody titre due to immunization with H1N1 vaccine. *The vaccine induced significant antibodies with single dose of vaccine after 21 days. **On booster dose the antibody level remained constant for the dilutions up to 28th day but showed some decrease in antibody titre on 40th day

observed for 40 days. On 21st day after primary inoculation, first group was bled by cardiac puncture and the sera were collected. Booster dose of vaccine was given to mice of other two batches. Then second group was bled on 28th day after the primary inoculation and 7 days after the booster dose while the third group was bled on 40th day after the primary inoculation and sera were collected separately. The inactivation of serum was performed by heat-periodate treatment method. Sera of separate group of mice were titrated for the HI antibodies through HAI test. The reference sera for the HAI test were obtained from CDC Atlanta and the antigen was standardized by back titration.

Hemagglutination inhibition (HAI) assay

The receptor destroying enzyme (RDE, CDC, Atlanta) treatment followed by 30 min heating at 56 °C in water-bath, was applied for the removal of 'non-specific inhibitors' from serum. Every serum sample was serially diluted in 25 µl PBS and then mixed with an equal volume of PBS containing 4 HAU of respective H3N2, H1N1 and Influenza type B virus. Incubated the plates for half an hour at room temperature and added 50 µl of 0.5 % RBC. The incubation was extended again for an hour. The resulting HAI titer was the inverse of the last dilution that inhibited agglutination.

Results

Characters of viral strains

For the isolation of virus, patients were selected on the basis of the presence or absence of influenza like illness (ILI) symptoms. The throat swabs and throat washings were collected from the patients and processed in the laboratory for isolation of the influenza virus. A total of 71 throat swabs from ILI cases were collected for the study and processed. The HA test was performed for virus detection in the clinical sample and all the strains were HA

positive (1:32). For further concentration of the virus the polyethylene glycol (PEG) method was used.

Hemagglutination inhibition test was performed for the identification of the H3N2, H1N1 and influenza B strain by using the respective reference Antisera obtained from WHO, CDC Atlanta. Antigen was identified on the basis of inhibition of hemagglutinin –RBC binding due to the presence of the antibodies.

The HAI results identified the strains as type A/Washington/15/91 like H3N2, type A/Taiwan/1/86 like H1N1 and B/Panama/45/90 like B type.

Virus inactivation

Repeated studies had been carried out to standardize the suitable temperature, time as well as the formalin concentration required for the proper inactivation of the viral vaccine strains. The HA titer was determined to check whether the strain is consistent or not. From the detailed studies summarized in Table 01(supplementary), it was concluded that as the titre of the virus remain constant after 4 h so that the time period of 4 h was adequate for complete inactivation of H3N2, H1N1, and B type influenza virus. Finally after the repeated tests, inactivation of the allantoic fluid containing the influenza virus was found to be effective with (0.03) % formalin for 4 h incubation at temperature of 37 °C.

Immunogenicity

Inactivated influenza vaccine including monovalent A/Washington/15/91 like H3N2, monovalent A/Taiwan/1/86 like H1N1, monovalent B/Panama/45/90 like B type and trivalent vaccine having all these three inactivated strains were used for carrying out the immunogenicity studies in the mice. The serum was raised as per the immunization protocol and then the HAI test was performed on the various sera (Table 02 supplementary).

After natural infection of influenza, normally serums anti hemagglutinin antibodies are developed in the body of

host. These antibodies are generally measured and correlated to the protection rate against influenza. A serum H.A.I. antibody titre of ≥ 40 can be detected in approximately 80 % of subject after natural infection [11] and this titre is considered to be protective against influenza virus infection [13]. The clinical diagnosis of infection is serologically confirmed, when a four-fold or greater rise in antibody titre can be shown to the types of influenza virus [12].

Immunogenicity for influenza monovalent vaccines

This vaccine induced significant antibodies titre with single dose of vaccine i.e. with 1:05, 1:10 and 1:25 diluted vaccine after 21 days. On booster dose the antibody level showed a fall in titre and the fall remain constant for each dilution on 28th day. After 40 days there was a rise in antibody titre and this rise was almost same to the antibody titre at 21st day. So results as shown in Table 02 (supplementary) and Fig. 1, it was made clear that the candidate monovalent H3N2 vaccine was able to produce the antibodies and was seroprotective.

The H1N1 vaccine induced significant antibodies with single dose of vaccine and showed the seroprotection. On booster dose the antibody level remained constant for the dilutions up to 28th day but showed some decrease in antibody titre on 40th day.

The type B vaccine induced significant antibodies with single dose of the vaccine. Also after the booster dose the antibody level showed a rise at 1:10 dilution and decrease at 1:25 dilution on 28th day but a constant level in antibody titre was seen on 40th day (Table 02 supplementary and Fig. 2). So it was clear that the vaccine was able to cause immunogenicity in the mice and antibody production was there due to dilutions and up to 28 days. Further on 40th day the level of antibodies fall down (Fig. 3).

Immunogenicity for trivalent influenza vaccine

The antibody response in the case of the H3N2 component of trivalent vaccine showed a rise with increase in the dilution and was immunogenic. The H1N1 part also showed a rise in antibodies with dilution up to 1:05 and after this it become constant. The same results were given by the B component. Hence the results showed that the vaccine was immunogenic and gave good immune response even at 28 days after the dose (Table 3 (supplementary), Fig. 4).

The immune response to the dilutions of trivalent vaccine was variable at 40th day after the primary immunization. Each component of the vaccine produced seroprotective antibodies in the mice as reflected in the HAI test. As the dilution of the vaccine increased, the HAI titre was also found to be in the ascending order. However all three components of the vaccine produced similar pattern of HAI titre at dilutions of 1:05 and 1:10.

Discussion

The influenza vaccines were produced as candidate vaccine in this trial study. The influenza virus strains were propagated in chick embryonated eggs. Centrifugation and filtration were done for the harvesting of the cultured virus. It is known that influenza viruses of prevalent strains are grown in the allantoic cavity of the embryonated hen's eggs. After incubation the allantoic and amniotic fluids are harvested. The virus is then pooled and inactivated by formalin or Beta-propiolactone. Then it is concentrated and purified. Then the vaccine is tested for its haemagglutinin content, neuraminidase content, protein content and sterility. The vaccine is then stored at (2–8) °C and supplied to distributors [14].

Fig. 3 Antibody titre due to immunization with influenza B vaccine. *The vaccine induced significant antibodies with single dose of vaccine after 21 days. **After booster dose the antibody level showed a rise at 1:10 dilution and decrease at 1:25 dilution on 28th day. ***On 40th day the level of antibodies showed decline

Fig. 4 Antibody titre due to immunization with trivalent vaccine. *Antibody titre was constant at 1:05, 1:10 trivalent vaccine dilution however there was downfall at 1:25 dilution

Whole virus vaccines generally stimulate the development of circulating antibodies to the envelope proteins of the virus. It has repeatedly been pointed out that viral proteins are retained during inactivation process to generate optimal production of antibodies. Formalin ($\geq 0.04\%$) can inactivate the virus effectively [15]. Formalin concentration from 0.01 to 0.07 % is able to inactivate the virus retaining its immunogenic properties. The time for the inactivation of the virus varies from 1 to 30 h according to the different concentration of the formalin used for the inactivation of the virus. Nowadays it is discovered that formalin treatment alone is not sufficient for the inactivation of the virus. So it is replaced with further treatment with detergents like Tween/ether/deoxycholate etc. [24]. On the basis of these studies the present study was undertaken and virus was inactivated by using 0.03 % of formalin applicable to monovalent as well as trivalent vaccines. The method of inactivation by 0.03 % formaldehyde in the study undoubtedly retained the envelope protein of influenza virus and conserved the biological activity of the same proteins as reflected in the hemagglutination titre. These results are also in line with the findings of Dorner et al., 2000 [16].

Inactivated influenza virus is when administrated intra peritoneally, induced high titers of neutralizing antibodies and stimulated a local IgA response. The intra peritoneal immunizations induced somewhat low titers as compared to the titer induced by the intranasal immunization. The immunogenicity was measured by hemagglutination inhibition titers. It means for better immunogenicity the virus must not only bind to mucosal cells but also fuse with the epithelial cells to stimulate optimal responses Martin and Bolwell 1991 [17].

When the formalized influenza vaccine is inoculated into mice by intra-peritoneal route, a humoral immune response is produced within 21 days and antibodies thus produced can be easily measured by the Hemagglutination Inhibition (HAI) test. Beside humoral antibodies, cellular

immune response (CMI) is also elicited [18]. The importance of CMI in the protection against infection with influenza virus has been studied by many workers. The present study is designed for the immunization of the mice on the basis of these findings.

It is concluded in this study that the candidate monovalent H3N2 vaccine was able to produce the antibodies and was seroprotective. It was clear that the monovalent H1N1 vaccine was effective in producing antibodies in whole time period with maximum antibody level on 21st day and due to 1:10 dilution of H1N1 vaccine. However an increase in the antibody titre was seen after twenty-one days of the booster dose. The reason might be that the residual and un-neutralized antigen would have triggered the production of antibody which was noticed on the 40th day. It is concluded that for effective immunization the optimum number of inactivated virus should be made available to produce maximum quantity of antibody. The overdose of vaccine containing more number of inactivated virus particles will somehow hamper the production of specific antibodies which needs to be investigated. The effect of booster dose was observed after 20 days of booster, which was established in this experiment.

A fall in the antibody titre was seen after the booster dose of the vaccine. This low level titre can be explained on the basis of neutralization of the new booster antigen by the circulating specific antibody already produced due to primary immunization.

In this study an approach towards the development of vaccine and calculating the HAI titre value was followed. It was ascertained that the monovalent and trivalent vaccines prepared using the isolates of A/Washington/15/91 like H3N2, A/Taiwan/1/86 like H1N1 and B/Panama/45/90 like B type were found to be antigenically effective based on the serological tests, pending purification to remove unwanted non-antigenic protein particles.

This study reveals that the present technology developed for the production of influenza vaccine using the influenza

isolates, can be developed further for large scale production of this vaccine, in case of any emergency. However purification of this vaccine will undoubtedly enhance the potency of the same vaccine. Hence the study involving different purification techniques needs to be undertaken to perfect the technology for the large scale production and testing of inactivated and potent influenza vaccines, taking in account the isolates prevalent in the identified area and as determined by W.H.O.

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