

# Protective Immunity in Mice Following Immunization with the Cochleate-Based Subunit Influenza Vaccines

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## ABSTRACT

High morbidity and mortality of influenza virus infection makes it an important disease world-wide. Mouse is a very well-studied animal model for this disease with similar manifestation to human disease. It would be desirable to induce mucosal as well as circulating immune responses to obtain protection from infection and to decrease the spread of the virus. Cell mediated immunity (proliferative and cytolytic responses) is needed for long-term immunity. A new type of influenza subunit vaccine which can be given orally or parenterally has been developed to induce mucosal and circulating immune responses. This vaccine consists of membrane proteins rolled up in a unique phospholipid structures called protein cochleates. BALB/c mice were immunized three times with influenza glycoprotein-containing cochleates orally or intramuscularly, or they were primed orally or intramuscularly followed by two boosts with the alternate routes. Proliferation assays of spleen cells and ELISA for IgA, IgG, and IgM of the sera and saliva from these mice shown some differences in the immune responses induced by different immunization regimens. Mucosal administration of the formulation led to secretory IgA in saliva while parenteral immunization resulted in circulating IgG. Increased proliferative responses as well as IgG following 2nd and 3rd administration, indicated the effect of boosting in all immunization regimens. Oral immunization with influenza virus envelope glycoprotein-containing cochleates led to protection from infection in the lungs and trachea following intranasal challenge with the live virus. *Iran. Biomed. J. 5 (1): 33-38, 2001*

**Keywords:** Influenza virus, Cochleates, Immunization

## INTRODUCTION

Influenza is a myxovirus from Orthomyxoviridae which causes acute infection of the respiratory tract and serious morbidity and significant mortality amongst the young, old and immunocompromised individuals on an annual basis world-wide [1, 2]. Because of the antigenic drift and shift of influenza virus, long-term protective vaccine has not been developed yet. As in the other viral infections, cell mediated immunity and circulating IgG in lungs are required viral clearance from the lungs and prevention of viral pneumonia [3]. Protective humoral responses are directed mainly against the receptor binding portion of the hemagglutinin (HA) surface glycoprotein [1, 2, 4]. Mucous membranes are the primary routes of entry for a wide variety of human disease-causing

agents, including those which are inhaled, ingested, or sexually transmitted. Mucosal (secretory) IgA is the major mucosal immune response to influenza virus in immunocompetent mice [5, 6]. Intramuscular (i.m.) or subcutaneous (s.c.) administration of the vaccines does not often lead to optimal or long lasting protection against these infectious agents [7]. In contrast, the oral route of delivery can stimulate strong protective responses on mucous membranes and in the circulation [8-11].

A new type of subunit vaccine, called protein cochleate has been developed in our laboratories [12, 13]. In this study, we demonstrated that influenza virus envelope glycoprotein-cochleates (IEGC) administered orally and/or i.m. as immunogen, induces cell mediated immunity as well as humoral immune responses. The effect of administration route in the induction of mucosal as

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well as systemic immune responses is studied. These immune responses are correlated with protection from live virus infection.

## MATERIALS AND METHODS

**Mice.** Female BALB/c mice, 6-7 months old, were purchased from Charles River Breeding Laboratories (Wilmington, MA, USA).

**Viruses.** Influenza A virus A/PR/8 (H1N1) was propagated by inoculation in allantoic sac of 10-11-day-old chicken embryo in a concentration of about  $10^6$  particles/ml. After 72 h, the eggs were transferred from warm to cold room and the allantoic fluids were collected the next day. Viruses were isolated and purified by high speed centrifugation at 4°C, aliquoted and stored at -70°C until use [14].

**Preparation of influenza glycoprotein-containing cochleates.** The IEGC vaccine was prepared from influenza virus A/PR/8 (H1N1) by the method of Gold-Fogerite *et al.* [12-15]. Briefly, the purified viruses were used to extract viral envelope glycoprotein using  $\beta$ -D-octylglucopyranoside (OCG) as a detergent to break lipid bilayer. The extracted viral envelope glycoproteins were added to the phospholipid and cholesterol dried down on the wall of a Pyrex tube. The suspensions were dialyzed against (N-tris [Hydroxymethyl] methyl-2-aminoethanesulfonic acid (TES) buffer without  $\text{Ca}^{2+}$  overnight following another dialysis against TES buffer containing  $\text{Ca}^{2+}$  to get viral envelope glycoproteins-containing cochleates.

**Immunization.** Mice were immunized peroral or intramuscularly (i.m.) with 100  $\mu\text{l}$  of the IEGC vaccine containing 50  $\mu\text{g}$  of glycoproteins for high dose and 6.25  $\mu\text{g}$  for low dose. The second immunization was with full dose and the third one was with 1/4 of the original dose. In the oral administration, 0.1 ml vaccine gradually dispensed into the mouth using a round drinking needle and allowing it to be swallowed. For i.m. immunization, 0.1 ml of protein-cochleates was injected into the thigh muscle of the left leg of the mice.

**Proliferation assay.** Mice were sacrificed and the spleen cells ( $1 \times 10^6$  cells/ml) were cultured in RPMI-10 containing 50  $\mu\text{M}$  2-mercaptoethanol (2-ME). One ml of the splenocytes was added to three

wells of a flat-bottom, 96-well sterile tissue culture plate. To obtain different concentration of the stimulant, different amounts of media were added to the proper wells. The cells were cocultured with the UV-irradiated influenza virus, Con A or media alone and incubated at 37°C.  $^3\text{H-TdR}$  (Dupont, Wilmington, DE) at 1  $\mu\text{Ci/well}$  was added to the culture 16-18 h prior to harvesting. Cells were harvested and after adding Ecoscint H (scintillation liquid, Kimberly Research, Atlanta GA) to the vials, the  $^3\text{H-TdR}$  uptake was determined using a  $\beta$ -counter [16].

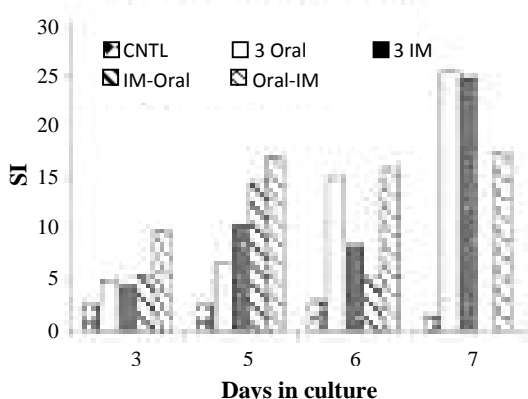
**ELISA.** Antibody to influenza virus glycoprotein was determined by ELISA using a protocol adapted from Klein-Schneegans *et al.* [17]. Immulon 2-flat-bottom 96-well plates were coated using 50  $\mu\text{l}$  of the extracted influenza virus glycoprotein at 6 mg/ml in carbonate-bicarbonate buffer at 37°C for 2 h. The plates were blocked with 1% BSA and then 50  $\mu\text{l}$  of mouse serum was added. For positive control, anti-influenza-positive sera were used. After washing, biotin-conjugated goat anti-mouse heavy chain specific antibody (purchased from Southern Biotechnology Assoc. Inc., Birmingham, AL 35209) was added. The addition of avidin-alkaline phosphatase (Sigma A 2527) was followed by the incubation at 37°C for 1 h. P-nitrophenyl phosphate (NPP, Sigma) was added as substrate and color reaction was measured.

**Determination of viral replication in lungs and trachea.** A group of the naive or immunized mice were challenged intranasally with  $10^9$  particles/25  $\mu\text{l}$  of live H1N1 influenza A virus. After three days, they were sacrificed and lungs and trachea were taken out and ground aseptically in sterile PBS, then sonicated. Cells and tissue debris were removed by pelleting at 2,500 rpm at 5°C for 30 min. Lungs and trachea extracts were inoculated in embryonated eggs. The inoculated eggs were kept at 35-37°C for 72 h. Allantoic fluids from the inoculated eggs were collected and assayed for virus presence by the hemagglutination assay (HA). Negative hemagglutination indicated the lack of influenza virus in the extract.

**Statistical analysis.** Experimental results were compared and analyzed using either the paired Student's *t*-test, ANOVA, Likelihood ratio test, or Fisher's Exact Test (2-tail).  $P < 0.05$  was regarded statistically significant.

## RESULTS

**Splenocyte proliferative response assay in the mice immunized with influenza envelope glycoprotein-cochleates by systemic vs. mucosal route of administration.** Proliferative responses were assessed in the spleen cells following immunization with cochleates containing influenza envelope glycoproteins. Mice were immunized with a full dose of glycoprotein cochleates (50 µg) at zero and 3 weeks and 1/4 dose at 13 weeks. Peroral or intramuscularly immunized mice as well as mice immunized by alternate routes showed strong proliferative responses to *in vitro* stimulation with UV-irradiated influenza virus (Fig. 1). The Con A stimulation of the naive as well as the immunized mice resulted in comparable proliferation with 250,000-375,000 CPM on day four of the culture (data not shown).



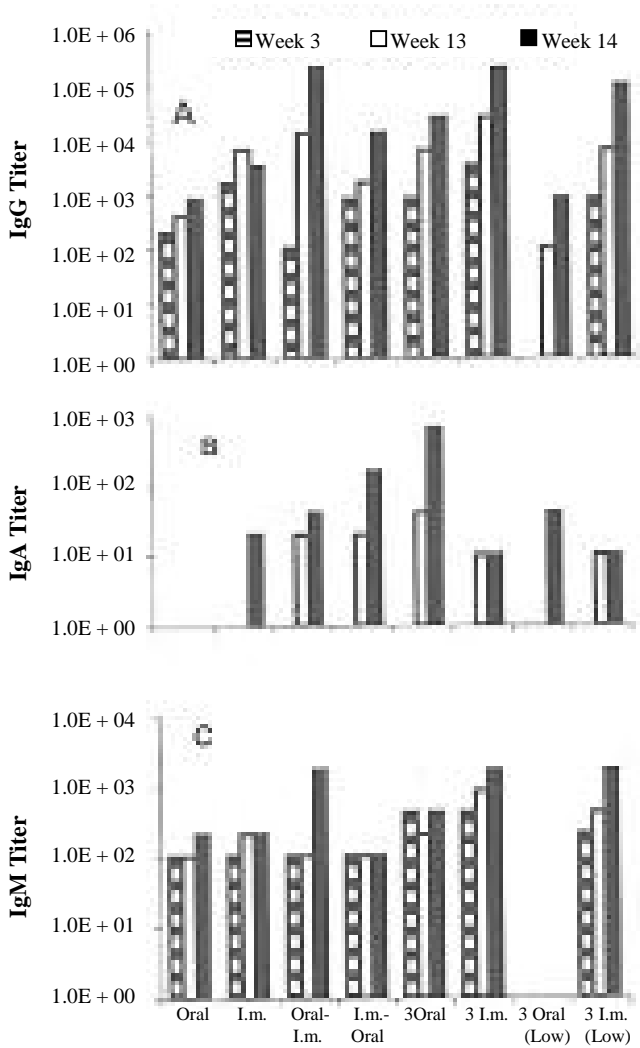
**Fig. 1.** Proliferation of splenocytes of immunized mice with cochleates vs. naive mice, after *in vitro* stimulation of UV-inactivated Influenza virus at 20 µg/ml was studied by measuring <sup>3</sup>H-TdR uptake at different time points after stimulation. Female BALB/c mice were immunized at week 0 and 3 with 50 µg and at week 13 with 12.5 µg Influenza-cochleates by 3 Oral or 3 IM or combination of alternate routes, and sacrificed at week 14. Stimulation index (SI) was calculated by dividing antigen-stimulated CPM by non-stimulated CPM. CPM of non-stimulated cultures were at the range of 700-1200.

The stimulation Index (SI) of the immunized mice were 3-12 fold more than the naive mice. The high proliferative response of the spleen cells of the mice immunized systemically or mucosally with cochleates indicated that this formulation efficiently induced cell mediated immunity in the mice immunized by both routes. The kinetics of the proliferative responses in the single route vs.

combinations was different. The single route-immunized mice mostly had a pick on day 7; in contrast, the combined routes-immunized mice mostly had the highest SI on day 5 of *in vitro* culture.

**Serum Igitotyping of the mice immunized with influenza cochleates.** Mice immunized with influenza cochleates were bled before and after each immunization and sera were assayed by ELISA. High titers of Ig's were obtained following immunization with high (50 µg) and low (6.25 µg) dose. Single immunization of mice with influenza cochleates resulted in higher circulating IgG titer in i.m.-immunized mice (as high as 6,400) than in the oral-immunized ones (the highest titer of 800) (Fig.2A). Anti-influenza glycoprotein (α-GP) IgG titers were 204,800 and 102,400 in the mice immunized three times i.m. and 25,600 and 800 in the mice immunized 3-oral with high and low doses, respectively (Fig.2A). In contrast, significant titer of α-GP IgA (as high as 640) was seen in mice immunized 3 times orally but not in 3-i.m. immunized ones (Fig. 2B). The combined-route immunization resulted in comparable antibody responses. (Fig. 2) The Ig titers were increased significantly after each immunization. The IgA titer was proportional to the number of oral immunizations; therefore the more mucosal administration of Ag, the higher circulating IgA titer. IgM titers were almost constant and low (Fig. 2C), suggesting that this immunization regimen efficiently induced Ig isotype switching and secondary antibody response upon boosting. There was no detectable Ig in the naive mice.

**Salivary Igitotyping.** Mice immunized once with IEGC peroral or i.m. and the mice triply immunized with low dose intramuscularly or orally, were boosted with 1/4 dose at week 53. Saliva of these mice was collected prior, and one and two weeks after the last boosting. The i.m.-immunized mice with high and low dose immunogen had IgG titer of 10 and 5, respectively with no detectable IgA. The mice immunized orally with high and low doses had IgA titer of 20 and 5, respectively, but no detectable IgG. Systemic immunization induced IgG but not IgA, in contrast, mucosal administration of cochleates induced good titer of IgA but not IgG. There was no detectable Ig in the saliva from the naive mice.



**Fig. 2.** Serum Igitypes in mice following immunization with Influenza glycoprotein-cochleates. Mice were immunized at week 0 and 3 with 50  $\mu$ g for high dose and 6.25  $\mu$ g for low dose (Low), and at week 13 with 1/4 primary dose of Influenza envelope glycoproteins in cochleates by the routes indicated. They were bled at listed times and ELISA for IgM (A), IgG (B), and IgA (C) was done on pooled sera of each group.

**Detection of viral replication in mice respiratory tract.** To scrutinize the protective effect of different doses of the immunization, the mice were immunized peroral with different doses of influenza glycoprotein cochleates, and then were challenged with live virus. The lungs and trachea of the challenged mice were taken out after three days. The lungs and trachea extracts were inoculated in 11-day-old embryonated eggs. Hemagglutination assay of the collected alantoic fluids determined presence of virus in the respiratory tract of all the non-immunized mice. The mice immunized with the highest dose were almost 100% protected. The

percent of protected mice was proportional to the dose of cochleates (Table 1).

## DISCUSSION

The new formulation of immunogen has been developed in our laboratory. It is called protein-cochleate consisting of protein of the interest inserted in lipid bilayer and rolled up by calcium bridges. Calcium ions in the last two dialysis buffer, bridges the negative phosphate groups of the phospholipids making roll up of lipid bilayer. Protein cochleates can be used to facilitate elucidation of the factors regulating the induction of the immune responses and correlative protection. They are highly effective immunogens when administered intramuscularly, resulting in strong circulating and mucosal antibody and proliferative responses to the viral glycoproteins. A long-term persistence of the immune response indicates a high probability of slow-release characteristics for protein cochleate formulations which will be consistent with their solid multilayered structure. In this study, proteins of interest were envelope glycoproteins of influenza virus, hemagglutinin and neuraminidase. The role of immunization route and the number of vaccinations on the kinetics, magnitude, and the type of attendant humoral and cellular immune responses have been investigated. A week after the first immunization, 2-3 fold increase in proliferative responses was seen in mice immunized orally or i.m. in comparison to the naive mice (data not shown). Strong proliferative responses are induced in the spleens of the mice immunized three times by systemic or mucosal routes (Fig. 1). Alternate priming and boosting also induced strong proliferative response in spleen but the kinetics of the response was different from single route immunization (Fig. 1). Influenza glycoprotein cochleate formulation induces comparable proliferative responses in single or combined immunized routes with different kinetics. Significant mucosal and circulating antibody responses induced by this formulation indicate the immunogenicity of the protein cochleate administered mucosally or systemically. The oral route gave very strong circulating IgG titers (25,600 at 14 weeks). Intramuscular immunization gave higher circulating IgG level. Oral immunization with the protein cochleate led to antigen-specific IgA in the saliva. In contrast, intramuscular immunization resulted in IgG only in the saliva,

**Table 1.** Protection study of mice immunized peroral with influenza envelope glycoproteins cochleates. Mice were immunized at week 0 and 3 with full dose and at week 13 with 1/4 dose. They were challenged intranasally at week 14 with 50 µg of live Influenza virus at  $2.5 \times 10^9$  particles/ml. They were sacrificed three days later and lungs and tracheas were taken out aseptically. The lungs and trachea extracts were inoculated in 10-11 days embryonated eggs, incubated at 37°C for 3 days. The positive HA of analectic fluids from both inoculated eggs per dilution indicated positive viral replication in respiratory tract of the mouse. Lungs<sup>1</sup>Mice from two separate experiments.

| Vaccine Dose (µg Protein /100µl Coch.) | Trachea # Infected/Total | Lungs # Infected/Total | Lungs <sup>1</sup> # Infected/Total |
|--|--------------------------|------------------------|-------------------------------------|
| 100.00                                 | 0/5                      | 0/5                    | 0/5                                 |
| 50.00                                  | 2/5                      | 0/5                    | 2/10                                |
| 25.00                                  | 0/5                      | 0/5                    | 1/10                                |
| 12.50                                  | 1/5                      | 0/5                    | 1/10                                |
| 6.25                                   | 0/5                      | 5/5                    | 6/10                                |
| 3.12                                   | 4/5                      | 5/5                    | 5/5                                 |
| 0.00                                   | 5/5                      | 5/5                    | 9/10                                |

probably as a result of transudation from the circulation.

In most vaccination systems, a combination of immunizing routes leads to superior immune responses and protection [18]. In contrast to most subunit vaccines, cochleates containing glycoproteins from influenza do not require priming parenterally [19]. Mucosal administration of the immunogen led to significant titer of circulating as well as secretory IgA, in comparison to, systemic immunization. To determine the effects of the combining alternate routes of the antigen presentation, mice were primed either orally or intramuscularly then boosted twice by the alternate route. Three oral-immunized mice were superior to i.m.-primed followed by two oral-boosted mice, both in terms of antibody and proliferative responses. In contrast, an oral prime with i.m. boosts gave comparable circulating IgG titers and somewhat higher cellular responses than obtained with 3 IM doses. Circulating IgA titer was proportional to the number of oral immunizations given. The more mucosal administration of protein cochleate the higher titer of IgA. It should be noted that there was no evidence of systemic tolerance following oral administration using influenza cochleates. Indeed, slowly rising antibody levels even after a single immunization indicates that tolerance is not occurring with this antigenic formulation. Obtaining higher responses with larger doses, and boosting of circulating antibody and proliferative responses exclude the theory of tolerance for this immunization regimen. Oral tolerance induction usually requires chronic dosing of larger quantities of the antigen [8, 20].

To elucidate the correlation of the protection with the immune responses induced, the orally

immunized mice with different doses of influenza glycoprotein cochleates were challenged with live virus. Viral replication in lungs and trachea of these mice was determined using hemagglutination assay of the inoculated eggs with lung and trachea extracts. In contrast to the non-immunized mice, the mice immunized with protein cochleates were protected from influenza infection in a dose-dependent manner (Table 1).

The cochleate is a new unique formulation of Ag which can be used for mucosal as well as parenteral administration to study the induction of the systemic and mucosal immune responses to variable immunogens including human disease causing agents. These and other studies show that the mucosal and systemic immunization with cochleates containing only the influenza virus glycoproteins and pure nontoxic, non-inflammatory phospholipids, stimulates strong, long-lasting circulating and mucosal antibody responses. They are highly effective immunogens when administered orally or intramuscularly and may be used for the development of the safe and efficacious vaccines for human.

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