Development of New Vaccine－New Method to Prepare Artificial Membrane Vaccine using Inter-Membrane Protein Transfer

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Abstract: Influenza is one of the common infectious diseases caused by influenza virus. Vaccines are the most effective means to fight infectious diseases. While these vaccines are quite effective in young adults, however, they are less for the above high risk group. A currently used influenza vaccine contains aluminum hydroxide as an adjuvant, which sometimes induces the production of IgE antibodies causing an allergic reaction. In order to overcome the drawbacks, artificial membrane vaccines or liposome vaccines have been developed. Liposomes are known to be effective immunoadjuvants. On the other hand, various membrane proteins, including matrix membrane proteins, have been reported to be spontaneously transferrable from living cell membranes to artificial membranes (liposomes). In this article, we introduced a new method to prepare the artificial membrane vaccine, "influenza virosomes", using inter-membrane protein transfer, and discussed the immunreactivity of the virosomes. We concluded that the application of inter-membrane protein transfer technique is a useful method for the preparation of the artificial membrane vaccine, virosomes. The virosomes showed high immunoreactivity especially with MDP (muramyldipeptide) derivatives as an adjuvant or booster treatment.

Keywords: Influenza, vaccine, liposomes, virosomes, inter-membrone protein transfer.

INTRODUCTION

Influenza is one of the common infectious diseases, which is caused by influenza virus and spread mainly in the late fall and winter. It differs from the common cold in high fever, headaches and extreme exhaustion. It is possible to become mortal disease for high risk group including the elderly, infants and the patients suffering from chronic diseases [1,2]. It is said that in the United States of America more than 200,000 people are hospitalized and about 36,000 people die from influenza every year. Vaccines are the most effective means to fight infectious diseases. Commonly used influenza vaccines are inactivated whole virions, split virions or subunit antigen vaccine. While these vaccines are quite effective in young adults, however, they are less for the above high risk group [3]. It has been known that the more purified the antigenic proteins are, the less effective the antigenic activity becomes [4-7]. To enhance antigenic activity Alum (aluminum hydroxide) has been used as an adjuvant, but Alum is known to induce production of IgE antibodies which often causes allergic reaction [8]. On the other hand, it has been found that liposomes can be effective immunoadjuvants [9-14]. In order to overcome the drawbacks mentioned above, artificial membrane vaccines or liposome vaccines have been developed [15-17].

1) ARTIFICIAL MEMBRANE VACCINE OR LIPOSOME VACCINE

Artificial membrane vaccines have shown high antibody titers against hemmaglutinin (HA) compared to the currently used influenza vaccine and have low side effects. Clinical trials have also shown high immune response even in elderly people [18,20]. Thus, artificial membrane vaccines are expected to be one of the most promising new types of vaccines. It has been reported that different modes of antigen association with liposomes, encapsulated and surface-linked, show different immunoreactivity [21,22]. Surface-linked liposomal peptide has been shown to serves as an effective vaccine without showing detrimental effects in the presence of immune potentiators [23,24]. Furthermore, influenza virosomes enhanced CTL (cytotoxic T lymphocyte) induction [25-27].

To reconstitute a transmembrane protein of cells on an artificial membrane, various detergents or organic solvents have generally been used. However, treatment with detergents or organic solvents possibly causes denaturation and inactivation of the target protein. In addition, the antigens were equally incorporated into the outer and inner leaflets of the liposomes by the currently used reconstitution methods. This has resulted in the reduction of the advantage of using liposomal vaccines. Recently, it has been reported that various membrane proteins are transferred spontaneously from cell to liposomal membranes without the solubilization and reconstitution steps using detergents or organic solvents [29-42].

2) SPONTANEOUS TRANSFER OF MEMBRANE PROTEINS FROM CELLS TO LIPOSOMES.

In early years, transfer of cytochrome b_5, which binds to the membrane surface due to its hydrophobic tail, was studied by many investigators [29-34]. This protein was considered to pass from cells to liposomes in the following manners: the protein is released into an aqueous phase from the cell membrane as monomer, and is transferred to

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liposomes, favorably to small unilamellar vesicles rather than to large vesicles. Huestis and his coworkers' studies focused on the transfer of more integrated proteins of cell membranes [35-37]. They reported that band 3 protein could transfer spontaneously from erythrocytes to liposomes, and the transferred band 3 protein in liposomes held a similar orientation in lipid bilayer as in the erythrocytes and original activity. In order to elucidate the above phenomena, they proposed a model where in transient fusion occurred between the cell membrane and liposomal membrane [37]. Furthermore they reported that inter-membrane protein transfer was enhanced by perturbing the lipid environment around the protein or membrane defect of donor cells caused by the penetration of recipient lipids or surfactant such as cholate, and that acetylcholine esterase transfers more to liposomes when the recipient membrane is more fluid than the donor [35-41]. Sunamoto and his coworkers reported that the addition of an artificial lipid 1,2-dimyristoylamido-1,2-deoxyphosphatidylcholine (DDPC) into phosphatidylcholine (PC) liposomes enhanced protein transfer from biological membranes such as erythrocytes and platelets [39,40,42]. They considered that the reason for the effect of DDPC on protein transfer was the enhancement of the holding ability of the transferred protein into the recipient membrane by intermolecular hydrogen bonding of those lipids [43,44]. Spontaneous protein transfer was not restricted to that from cells to liposomes. We found that HA protein on the CV-1 cells infected with influenza virus could transfer to erythrocyte membrane ghosts [45]. We succeeded in a protein (superoxide dismutase) delivery from the protein-encapsulated erythrocyte ghost to living cells (CV-1 and HeLa cells) by taking advantage of the fusing ability of the HA protein incorporated into the erythrocyte ghost membranes [46-48]. Recently, Niu *et al*., reported that a vriety of transmembrane proteins and other cellular components are transferable between multiple cell types, which were induced by transient local membrane fusion [49]. Thus, many investigations have been done about spontaneous membrane-protein transfer from cell membranes to artificial membranes such as liposomes, or cell to cell. On the other hand, there are some reports which refute spontaneous protein transfer, such as how no spontaneous transfer of the GPI-linked protein occurs at all [50]. Although clear explanation for the mechanism of spontaneous protein transfer from biological membranes to liposomes and its regulating factors have not been established yet, this idea has much possibility as a new method of reconstituting membrane proteins on artificial membrane. We started to study on the development of new method to prepare liposome vaccines, virosomes, against the influenza virus adopting the application of the inter-membrane protein transfer.

3) CONSTRUCTION OF INFLUENZA VIROSOMES USING SPONTANEOUS PROTEIN TRANSFER AND THEIR BIOLOGICAL ACTIVITY AS AN ARTIFICIAL MEMBRANE VACCINE

Experimental procedures [51] were as follows: CV-1 cells from monkey kidney were infected with influenza virus (A/NWS/33, H1N1 subtype). CV-1 celles and the virus were obtained from Denka Seiken Co. Ltd. (Niigata, Japan). After incubation at 37^{o}C for 20-24h, liposomes were added and further incubated for proper time. The liposomes were recovered and the transferred antigenic proteins HA and NA (neuraminidase) were monitored. Immunoactivity of HA, NA-incorporated liposomes (virosomes or artificial membrane vaccine) was evaluated.

First, how much protein could be recovered on the liposomal membrane in all released protein was studied. In higher temperature more proteins [51, 52] were released. The recovered protein seemed to be almost proportional to the released protein in each temperature and was about 20 % of all released protein [51]. The transfer efficiency was influenced by several factors, such as lipid composition of acceptor liposomes, liposome size, incubation temperature and so on. For recipient liposome size, the recovered protein was highest on SUV (small unilamellar vesicle) [53] in accord with the previous report regarding transfer of cyto-chrome b_5 [34-34]. For lipid composition, the incorporation of DCP (dicetylphosphate), DPPS (dipalmitoylhosphatidylserine) and DMDPA (dimethyldipalmitoylammonium) into DMPC (dimyristoylphosphatidylcholine) liposomes enhanced protein transfer. On the other hand DPPC (dipalmitoylphos-phatidylcholine) and bbPS (bovine brain phosphatidylserine) had no effect on protein transfer. DCP and DPPS were anionic lipids, and DMDPA was a cationic lipid. DPPS and bbPS have the same head group [54]. These results show that electrostatic condition does not significantly affect the protein transfer, consisting with a report of Waters *et al.,* [41]. Instead, the phase separation of recipient vesicles might enhance inter-membrane protein transfer allow for the common property of the membranes composed of mixture of DMPC and DCP, DMPC and DPPS or DMPC and DMDPA which was in the state of phase separation at 37℃ [55]. In addition, membrane fluidity did not crucially affect protein transfer [54]. Two influenza virus-specific proteins, HA and NA, which were present on the membrane of virus-infected CV-1 cells, were also transferred from the cell membrane to liposomes as shown in the Western blotting[53] and SDS (sodium dodecyl sulfate)-PAGE (polyacrylamid gel electrophoresis) after immunoprecipitation [52]. As shown in Fig. (**1**), three proteins, HA1, HA2 and NA, were detected. In order to know the distribution and localization of transferred viral proteins on the liposomal membranes, fluorescent images of the liposomes were observed using FITC (fluorescein isothiocyanate)-labeled antibody and freeze-fracture electron micrograph. RITC (rhodamine B isothiocyanate) dextran-incorporated REV (reverse-phase evaporation vesicle) liposomes were incubated with the influenza virus infected-CV-1 cells, and the liposomes were collected by centrifugation. The liposomes were treated with a primary antibody (rabbit anti-influenza virus antibody) followed by a secondary antibody (FITC labeled anti-rabbit IgG antibody). As shown in Fig. (**2**) [56], the fluorescences of RITC and FITC were observed throughout entire liposomes, suggesting that transferred antigenic proteins were distributed all over the membrane surfaces of the liposomes. As shown in Fig. (**3**) [57], spikes of viral proteins on the liposomal membranes were observed just like influenza virus particles on freeze fracture electron micrograph, suggesting that transferred viral proteins were aligned in a similar manner as influenza virus particles. In searching for the mechanism of inter-membrane protein transfer, two pathways were assumed: one was direct transfer by contact of liposomal membranes with cell

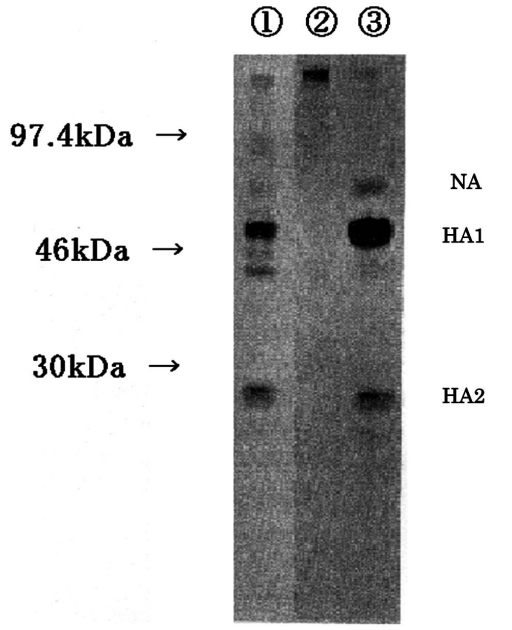

Fig. (1). Detection of influenza virus glycoproteins by SDS PAGE after immunoprecipitation. Lane 1: influenza virus-infected CV-1 cells. Lane 2: liposomes incubated with CV-1 cells. Lane 3: liposomes incubated with influenza virus-infected CV-1 cells.

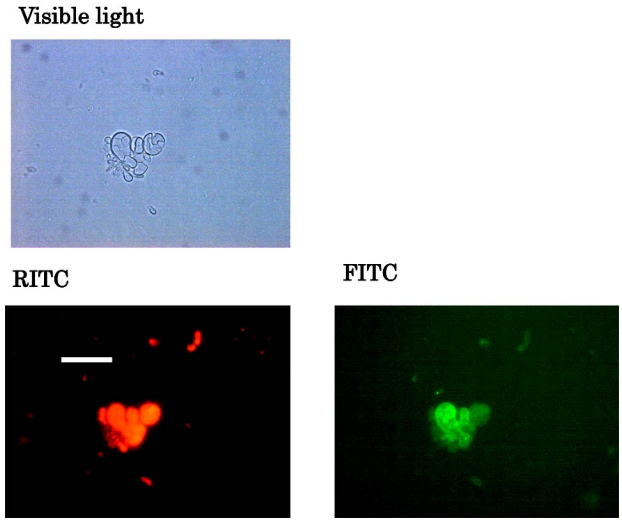

Fig. (2). Visible light and fluorescent images of REV (reverse-phase evaporation vesicle) liposomes incubated with influenza virus-infected CV-1 cells. Visible: vsible light image. RITC: fluorescent image of RITC-labeled dextran encapsulated in the liposomes. FITC: fluorescent image of FITC-labeled antibody (2^{nd} antibody).

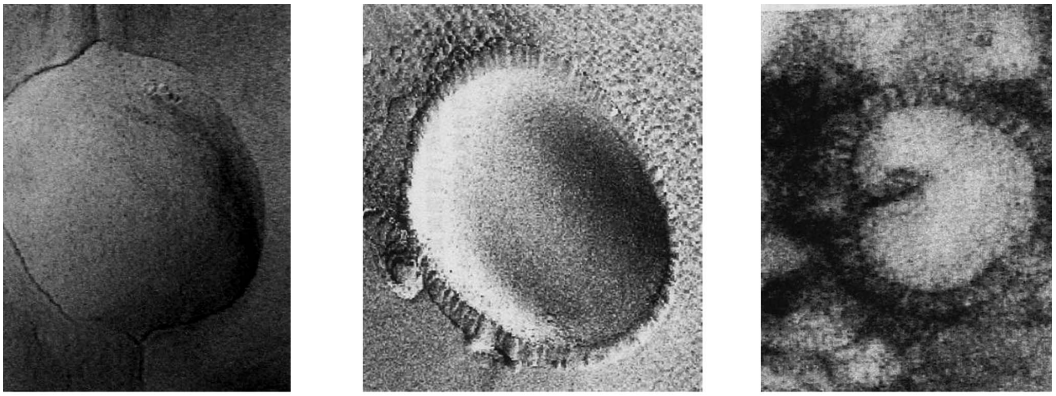

Fig. (3). Freeze-fracture electronmicrographs of control liposome and liposome incubated with infected cells; **A**) Original liposome before incubation; **B**) virosome after incubation with influenza virus-infected CV-1 cells; **C**) negative-staining electron-microrograph of influenza virus.

membranes (Huestis' model); the other was protein transfer from the medium to liposomes after proteins on the cell membrane had been released into the medium. In order to verify the latter case experimentally, the virus-infected cells were incubated in the medium (sorting buffer) in the absence of liposomes for 1 h at 37°C. After removing the cells by centrifugation the liposomes were added to the medium. The membrane proteins could be recovered into the liposomes in this experiment, although the quantity of transferred proteins in the latter case was about half of that in former case. The transferred proteins in the latter case were about one third of released protein. The virus specific proteins transferred from CV-1 cells to liposomal membrane was monitored by immuno precipitation. In both cases, a band, which was assigned to influenza virus protein (HA1), was observed on an autoradiograph image after immuno precipitation (Fig. **4**) [57]. Above results showed that there should be another pathway in addition to Huestis' model.

The above prepared proteoliposomes were injected intraperitoneally to BALB/c mice as an artificial membrane vaccine, and the titers of neutralizing antibodies in sera were measured at 1, 2 and 3 weeks after immunization [58]. The booster effect was also examined by injecting the artificial membrane vaccine 2 weeks after the first immunization. The results were shown in Fig. (**5**) [58]. After 1 week of immunization, antibody titer in mice treated with the inactivated virus (positive control group) was higher than that of the positive liposomes (viral protein-incorporated liposomes). However, 2 weeks after immunization, antibody

titers in the positive liposome group were comparable to that of positive control group, especially, after booster treatment. In another experiment, a muramyl dipeptide derivative, B30-MDP, was added as an adjuvant in the liposome preparation, potent adjuvant activity was observed by B30-MDP in a dose-dependent manner (Fig. **6**) [58].

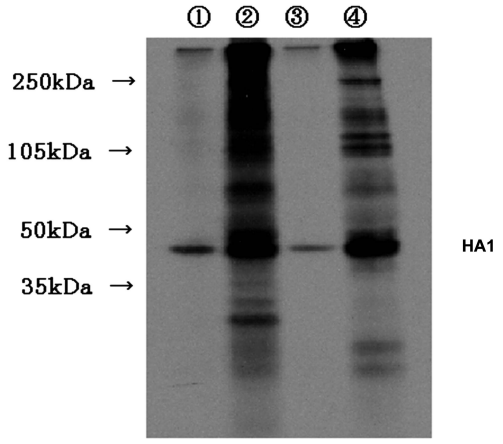

Fig. (4). Detection of influenza virus glycoproteins by SDS PAGE after immunoprecipitation in the different conditions. Lane 1: liposomes after incubation with influenza virus-infected cells; lane 2: infected cells; lane 3: liposomes after incubation with medium, which had been incubated with infected cells; lane 4: medium.

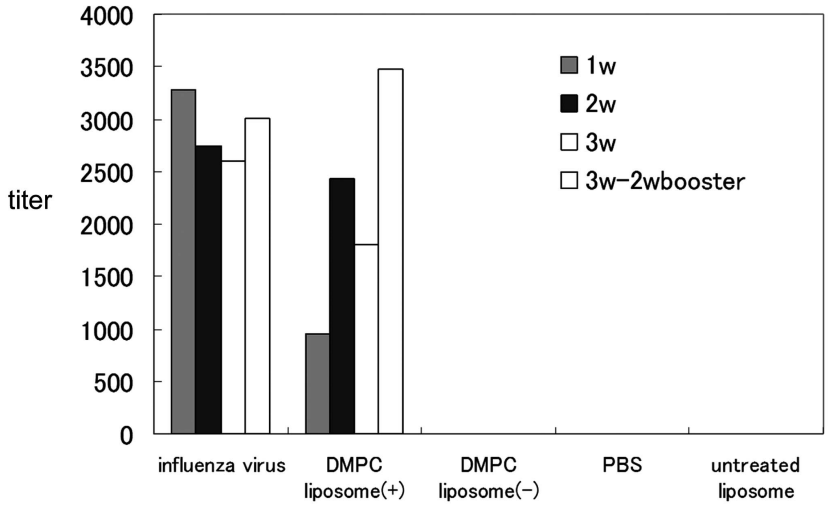

Fig. (5). Neutralizing antibody titers in sera of mice intraperitoneally administered with inactivated viruses or virosomes. DMPC Liposome(+) : liposomes incubated with infected cells; DMPC liposome(-) : liposomes incubated with non-infected cells untreated liposome: liposomes not incubated with cells.

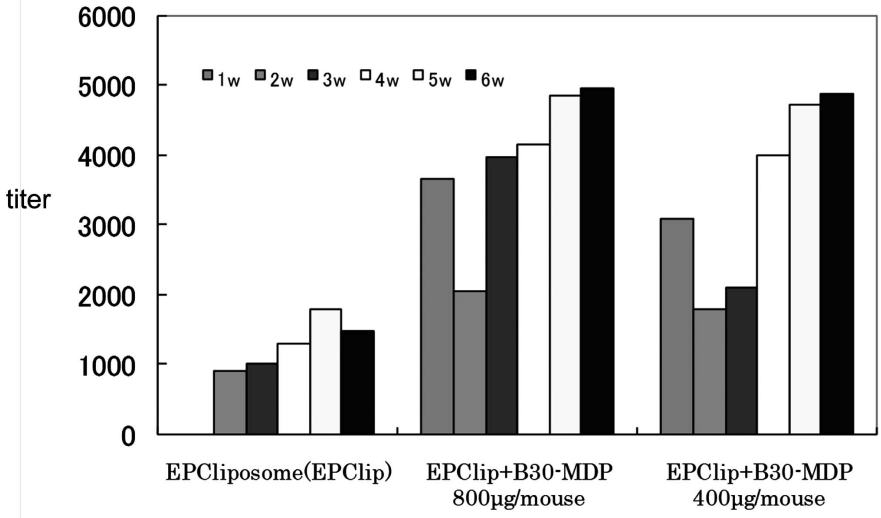

Fig. (6). Effect of B30-MDP on the production of virus-specific antibody. EPC liposomes: EPC (egg yolk phosphatidylcholine) liposomes without B30-MDP incubated with virus-infected CV-1 cells. EPC+B-30MDP: EPC liposomes containing B30-MDP.

Next, we compared the effects of administration routes of vaccines, that is, intranasal and subcutaneous administrations, on the production of virus-specific antibody. As the vaccines, we used inactivated influenza virus, liposomes incubated with influenza virus-infected CV-1 cells (virosomes), and the mixture of virosomes and B30-MDP at the same quantity of virus-specific glycoproteins (HA and NA). These vaccines were applied to BALB/c mice, and the neutralizing antibody against influenza virus was titrated in the sera collected 1 and 4 weeks after treatment with vaccines. As shown in Fig. (**7**) [56], the production of the antibody was observed in the mice treated with intranasal virosomes and the mixture of virosomes and B30-MDP as early as 1 week after vaccination, while the other groups showed very small levels of antibody. At 4 weeks of intranasal vaccination, the levels of antibody in the mice treated with the virosomes and the mixture were almost the same as that in the mice treated with inactivated virus. On the other hand, markedly high titers of antibody were obtained in the mice treated subcutaneously with the virosomes and the mixture as compared with that in inactivated virus-treated mice. From these results, it was found that virosomes could quickly stimulate the immune function of mice for the protection from virus infection.

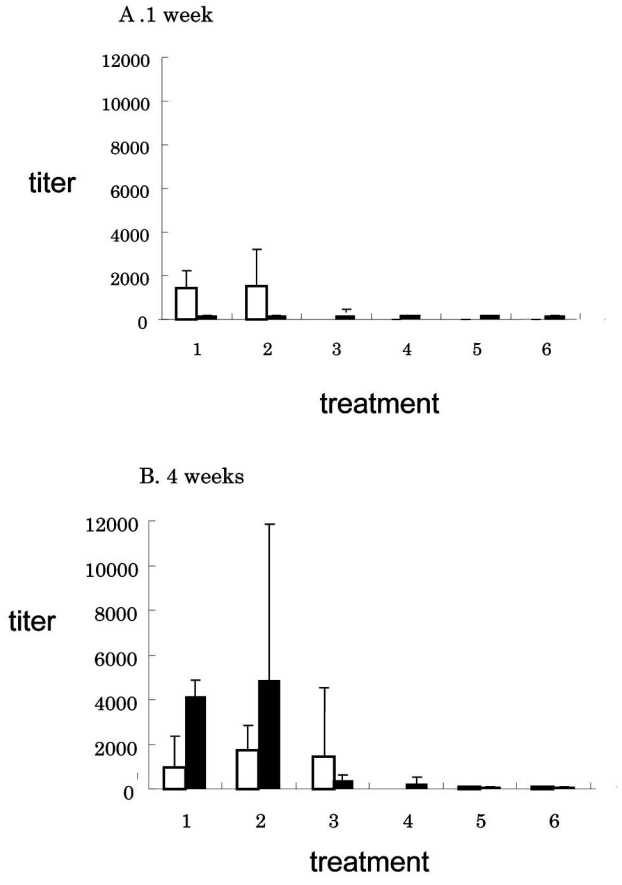

Fig. (7). Neutralizing antibody titers in sera 1 or 4 weeks after intranasal (open squares) and subcutaneous (closed squares) administration. 1. liposomes incubated with influenza virus-infected CV-1 cells (virosomes). 2. virosomes + B-30 MDP as an adjuvant. 3. inactivated virus. 4. liposomes incubated with uninfected CV-1 cell. 5. Liposomes. 6. PBS.

4. CONCLUSION

Using inter-membrane protein transfer is a useful method to prepare the artificial membrane vaccine, virosomes. The virosomes showed high immunoactivity especially with MDP derivatives or booster treatment.

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