行政院國家科學委員會專題計畫研究成果報告

A 型流行性感冒病毒 M2 蛋白質基因疫苗之研究

Study of M2-protein Based DNA Vaccine for Influenza A Virus Infection

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中 文 摘 要

A 型流行性感冒病毒其表面兩種醣蛋白質會進行抗原轉變及抗原飄變，使得抗原變異性相當明顯，目前預防流行性感冒之疫苗為去活化之病毒並以注射方式接種，此種疫苗不會激發黏膜免疫力，此與老年人較低之免疫效果應有關連。黏膜的 IgA 抗體與細胞免疫力對於預防流行性感冒都很重要，基因疫苗有潛力激發此兩種免疫反應，基因疫苗之發明對疫苗研發提供了一個全新的發展方向，同時也可以避免傳統疫苗的一些缺點。流行性感冒疫苗之成份每年均必須評估是否需要改變，至今廣效性的疫苗尚未發展出來。M2 蛋白質是流行性感冒病毒的一種穿膜蛋白質，在不同的 A 型流行性感冒病毒之間相似性高，本計畫以 M2 蛋白質為基礎，輔以細胞激素或免疫佐劑，期以發展出一種廣效的基因疫苗可以對抗不同亞型之 A 型流行性感冒病毒感染。

本計畫完成構築 M2 蛋白質與修飾過之 M2 蛋白質之 DNA 載體，結果經剔除穿膜部份之 M2 蛋白質可以成功的在大腸桿菌中表現蛋白質，並經 M2 單株抗體確認。小鼠之免疫實驗，以肌內方式注射帶有 M2 蛋白質基因之質體，結果顯示帶有全長的 M2 蛋白質基因可以刺激小鼠產生抗體反應，而經剔除穿膜部份之 M2 蛋白質基因卻未見此抗體反應。在體外之細胞實驗中，亦有相類似的結果，僅有全長的 M2 蛋白質基因會成功的於細胞中表現 M2 蛋白質。因此，另將剔除穿膜部份之 M2 蛋白質基因加以修飾，其兩端分別加上訊號胜太與 Fc 片段之序列，期以增強免疫反應，結果顯示確有增強免疫反應的效果。並在體外證明此與 Fc 片段結合之分子可與帶有 Fc 受器之小鼠巨噬細胞 P388D1 進行結合，此作用應有助於加強 M2 蛋白質之免疫效果。此外，免疫小鼠亦同時加入 IL-5 或 GMCSF 基因之質體，發現 IL-5 可增強免疫反應的效果，而 GMCSF 則不然。

本計畫已將結果寫成英文論文，如下所附，即將準備投稿。

關鍵詞：A 型流行性感冒病毒，基因疫苗，M2 蛋白質

ABSTRACT

Intramuscular injection is most often applied in DNA vaccination. Myocytes are the major targets to be transfected by this method. Because myocytes are nucleated cells with few MHC class I and no MHC class II on the cell membrane, it is considered to be an "antigen production factory". Increasing the production of antigen from transfected myocytes might be a way to enhance the immune response by DNA vaccine inoculation. In this study, we cloned the gene encoding the influenza A virus M2 protein deleted at transmembrane region, residues 25-55 (pMd). In order to enhance the expression by the host cell, signal peptide of *Staphylococcus aureus* protein A was fused to the N-terminus of the M2d25-55 (pSMd). Meanwhile, human IgG1 Fc fragment was fused to the C-terminus of M2d25-55 (pSMdFc) to target the antigen expressed to the antigen presenting cells. Moreover, Th2 cytokine, IL-5, was cloned and used as the adjuvant to augment the M2 specific IgA response. Using *E.coli* as the expression host to express MBP-MdFc, we demonstrated that Md antigen was indeed targeted to the murine macrophage cell line, P388D1, via the interaction of the fused Fc and Fc receptor on P388D1 cell. In the in vivo experiment, influenza A virus M2 specific antibody was detected in the mice immunized with pSMdFc alone or with pIL-5, however, none of the mice immunized with pMd or pSMd alone or with pIL-5 could be detected to be M2 specific antibody positive. In this study, we had successfully demonstrated the effectiveness of Fc fusion in the enhancement of M2 antibody response after administration of DNA vaccine. This showed the Fc fusion with the potential as an alternative approach to enhance the immune response differing from the applications of other traditional adjuvants.

Keywords: Influenza A virus, DNA vaccine, M2 protein

INTRODUCTION

Traditional vaccines of influenza virus provide protections by induction of neutralizing antibodies mainly against HA or NA. However, there is a major shortage of traditional vaccine that the antigenic shift between HA and NA can decrease the potency of traditional vaccines and needs repeated inoculation each year.

Compared to HA and NA, M2 is much more conserved among different subtypes of influenza A virus. The potential of M2 as an antigen for influenza vaccine has been addressed since 1988. All of them demonstrated that M2 seemed to be a suitable antigen of influenza virus vaccine by administration of M2 recombinant protein via intramuscular injection. Kenji et al. (12) use M gene of influenza A virus as an antigen and showing a significant protection in immunized mice from influenza virus lethal infection. Not only cellular immunity but also influenza specific antibody response was induced. It is known that antibody can mediate the cytotoxic effect through antibody-dependent cellular cytotoxicity, and monoclonal antibody of M2 has the ability to restrict influenza viral growth (11).

So far, there has been reported that recombinant M2 protein truncated at transmembrane portion can successfully elicit immune responses against influenza A virus challenge in mice (18). We want to know if this modification of M2 can also work in DNA vaccine. Therefore, we construct pMd which is truncated at amino acid 25-55. We also construct two other forms of truncated M2 DNA vaccine to achieve the optimal protection effect. We fused a signal peptide of *Staphylococcus aureus* protein A to the N-terminus of M2 deletion (25-55) to get pSMd. The signal peptide of *Staphylococcus aureus* protein A is known to enhance the secretibility of the fusion protein (15), therefore, the production and secretion of protein might be enhanced from the transfected myocytes. Furthermore, we fused a human IgG1 Fc fragment to C-terminus of M2 deletion (25-55) to get pSMdFc. The fusion of human IgG1 Fc

fragment is thought to target the antigen expressed by transfected myocytes to antigen presenting cells through the interaction between Fc of fusion protein with the Fc receptor expressed on the antigen presenting cells.

IL-5 can stimulate the differentiation of IgA^+ B cells, which dominantly exist in the mucosal area rather than muscle. Since influenza virus infect mucosal area of upper respiratory tract, using IL-5 as the adjuvant is likely to enhance the antigen specific IgA production and serves as the first defensive line for viral infection. Therefore, we construct pIL-5 as the adjuvant.

Using these approaches, we want to know the optimal construction of M2 DNA vaccine and the adjuvant combination, and hopefully to provide future application of influenza virus vaccines.

MATERIALS AND METHODS

PCR and Plasmid construction:

Full length murine IL-5 was amplified from pBS-SK(+)mIL-5 (kindly provided by Jane Olsen, ANU) by primer pair 1 (table.1). Signal peptide was amplified from signal sequence of *Staphylococcus aureous* protein A by primer pair 2. Truncated M2 cDNA were amplified by primer pair 3 from pMd which contains a M2 deleted at amino acid 25-55. Human IgG Fc portion was amplified from PBMC (peripheral blood mononuclear cell) by primer pair 4. Two constructs were prepared: (1) pSMd contains the signal peptide at N-terminus of truncated M2. (2) pSMdFc contains the signal peptide at N-terminus and human IgG1 Fc fragment at C-terminus of truncated M2. In order to maintain the conformation integrity of both M2d (25-55) and hIgG Fc, a linker coding for (GGGGS, G_4S_1) was inserted at the junction of both genes. Plasmids were transformed into competent E. coli strain JM109 and plasmid sequences were verified by automated nucleotide sequence (ABI373 DNA sequencer) analysis using standard protocols.

Recombinant M2Fc protein was expressed and used to confirm the targeting ability of the human IgG1 Fc fragment in vitro. M2Fc gene was amplified from plasmid pSMdFc by primer pair 5 and cloned into Bam HI/Xba I sites in pMAL-c2x (New England BioLabs) which contains a maltose binding protein at 5' multiple cloning sites, therefore it can produce a MBP-fusion protein. MBP can serve as a tag to facilitate purification of recombinant protein, MBP-M2Fc. After confirming the sequence of pMAL-M2Fc, BL21(DE3), pLys S (Novagen) was used as the competent cell and transformed by heat shock.

RNA expression by in vitro transfection:

pMd, pSMd, pSMdFc were used to transfect 293T cell by Lipofectamine 2000 (Invitrogen). For RNA extraction, cells were lysed by Trizol™ (Gibco-BRL) directly

on the plates after removing the medium. Extracted RNA was treated with DNase (Qiagen) to digest any possible DNA contamination, then divided into two parts. One for RT-PCR and the other undergo PCR directly as counterparts to exclude DNA contimination. SuperRT and SuperTaq were used at 2U/μl. Human placenta RNase Inhibitor was used at 5U/μl. Poly d(T)n (Applied Biosystems) was used at 2 pmol. Each PCR product was electrophoresised in 1.5% EtBr containing agarose gel.

SDS-PAGE and western blotting:

Expressed proteins were separated SDS-PAGE with 7.5% stacking gel and 17.5% running gel. Proteins were running under 120V constant voltage in stacking gel and 200V constant voltage at running gel. Gel was stained by 0.05% coomassie blue for 10 minutes and distained by distaining buffer overnight. For western blotting, protein was transferred to nitrocellulose membrane (NC membrane (Schleicher & Schuell, Germany)) by capillary method. 14C2 mAb was used as 1^{st} antibody at 1:5000 dilution and HRP conjugated goat anti-mouse IgG was used as 2^{nd} antibody at 1:500 dilution. Color was developed by DAB method.

Fc targeting assay:

MBP-M2 was was used as negative control for specific Fc targeting. For Fc-mediated binding, MBP-M2 or MBP-MdFc was incubated with $2x10^5$ P388D1 cells on ice for 1 hour to ensure no endocytosis occurred under this condition. For internalization assay, the incubated P388D1 cells were transferred to 37□ to allow membrane flow and endocytosis. The kinetics of ligand internalization was determined at 5, 15, 30 minutes after transferring of cells to 37□. At each time point, pellet the cells and wash the cells twice with PBS. Cells were then fixed by 3.5% paraformaldehyde at room temperature for 15 minutes. Then, resuspend the cells with 1.5% BSA in sterilized water and drop on slice. Targeting and internalization were detected by immunofluorescent method using 14C2 was 1^{st} antibody and FITC

conjugated goat $(Fab')_2$ anti-mouse IgG was 2^{nd} antibody. For counter stain, secondary antibody was diluted with even blue at 1:30. Results were visualized by fluorescence microscopy (Zeiss MGDS).

Measurement of murine IL-5 by ELISA:

In order to confirm the protein expression of pIL-5 in advance, Balb/c were intramuscularly injected 100μg pIL-5 at quadricepses. Quadricepses of mice were surgical ablated at day 1, 3, 5, and 7 and homogenated with liquid nitrogen, then dissolved in 500 μl sterilized water with 0.1M PMSF. Supernatant was collected after sonication and detected the expression of IL-5 protein by sandwich ELISA method with mIL-5 ELISA kit (Endogen) which using TRFK-5 as the capture protein and TRFK-4 as the detection protein. IL-5 quantity was measured by absorbance at OD_{450}-OD_{550}.

Animal vaccination and evaluation of antibody response by ELISA:

All mice were immunized at six to eight weeks of age and were grouped of five mice for each DNA construct. Mice were anesthetized by intraperitoneal of 30 μl aceppromazine maleate (Fermena Animal) before vaccination. Mice were immunized intramuscularly three times at two weeks interval of 100, 50, 25 μg DNA vaccine in normal saline into left and right quadriceps by 29-guage neddle and insulin suringe (Beckman Coulter). For pIL-5, it was mixed with each DNA construct at 1:1 ration and was given at the second and the third inoculation as the adjuvant. Mice were bled by tail one day before inoculation and continued being bled at two weeks interval will 14 weeks. Serum was collected after centrifugation at 5000g. Specific antibody titers were expressed as the reciprocals of the final detectable dilution values, which gave an OD_{490} > three folds of standard deviation of negative control.

Evaluate the antibody response by ELISA:

Antibody response was measured by ELISA with MBP-M2 or MBP as coading

proteins. HRP-Goat anti-Mouse IgG (1:5000) or HRP-Goat anti-Mouse IgG1/IgG2a (1:1000) were added as detection antibodies. Color was developed by OPD (o-Phenylenediamine, Sigmal) method. For IgA ELISA assay, serum was ten fold diluted and incubated with protein A to pre-clear all IgG in the serum. After centrifugation, supernatant was collected and added as the first antibody in ELISA assay and HRP-Goat anti-Mouse IgA was used as secondary antibody. Measure the absorbance at OD_{490}.

Nutralization test:

One day before NT assay, plating 4-5*10^5 per well MDCK cells on 6 well plates so that cells can grow to 100% confluence on day for experiment. Influenza A virus (A/PR/8/34, mouse adapted virus) was diluted by E-0 (minimum essential medium) with 10ng/ml trypsin which was identified by plaque assay to yield 30-40 plaques on MDCK grown in 6 well plates. Serum was first decomplement at 56□ for 30 minutes and was 2-fold diluted by E-0 with 10ng/ml trypsin from 1:4 to 1:32, and incubate with virus at same volume at 37□ for 1 hour. 14C2 was also tested as a positive control and diluted to 1:1000 (49) for this assay. One hour later, the antibody-virus mixture was transferred to MDCK and incubated for another one hour for viral absorption. Shake the plate every 15 minutes. Add the agarose mixture (3% agarose: 5 μg/ml trypsin E-0=1:9) to the MDCK. Two days later, cells were stained with 1% Neutral Red (Filtered) and fixed by 10% formalin 24 hours after staining.

RESULTS

Contruction of DNA vaccine and RNA expression by in vitro transfection 293T cell:

Each gene was amplified respectively and fused by $2^{nd}/3^{rd}$ round PCR and cloned to pcDNA (Fig.1). RNA expression of each plasmid was determined after transfected into 293T cells and detected by RT-PCR. Each fusion gene was successfully transcribed by mammalian cells (Fig.1).

Expression of MBP-M2Fc recombinant protein:

Recombinant M2-Fc fusion protein was expressed by *E. coli* and used to confirm the targeting ability of the human IgG1 Fc fragment. The signal peptide was removed to mimic the real situation in mammalian cell. The maximum expression was observed at the forth hour after induction and remained a plateau till the sixth. Protein expression and purification were then confirmed by Western blotting (Fig.2). The molecular weight of MBP is approximately 43 Kd and MBP-M2Fc is 77Kd. The conformation of the Fc fragment and M2d was intact after confirmed by native-PAGE. Both the Fc fragment and M2d remained their epitopes to HRP-goat anti-mouse IgG and 14C2, respectively under native PAGE (Fig.3). All together, these results suggested that the 14C2 epitope of M2d or the Fc conformation were not obstructed by the fusion construction.

Fc-mediated binding and internalization of MBP-M2Fc is visualized by fluorescence microscopy:

The percentage of total ligand-positive cells treated with MBP-M2Fc was approximately 10%. As compared to MBP-M2, MBP-M2Fc was much more efficient in targeting to P388D1 at 4 □ (Fig.4). MBP-M2 was not found to be internalized; however, MBP-M2Fc was internalized after binding at 4□ for 1 hour. MBP-M2Fc was not detected after 30 min, which indicated that the degradation of the ligand

internalized by Fc mediated endocytosis was quite rapidly.

Measurement of murine IL-5 by ELISA:

Murine IL-5 expressed from pIL-5 inoculation was determined by ELISA. One day after inoculation, IL-5 was detected at the amount of 0.4 μg per gram of quadriceps. The IL-5 level is similar at every time point, range from 0.4-0.6μg per gram of quadriceps. It seems that there is no positive relation between the time and quantity of protein expression.

M2 antibody response was elicited in mice immunized with pSMdFc alone or with pIL-5:

Next, we analyzed M2 antibody titers after DNA immunization. Serum collected from immunized mice and naïve mice were tested for the M2 titers of the IgG, IgG1, IgG2a and IgA subclass specific to influenza A virus M2 protein. IgG titer was induced in mice immunized with pSMdFc and pSMdFc + pIL-5, and the titers which was sustained for more than two months after the last immunization (Fig.5). The titer were significant difference ($p<0.02$) when compared to naïve mice. Moreover, mice immunized with adjuvant (pIL-5) produced a much higher Ab titers than those immunized with pSMdFc alone. However, no detectable M2 antibody titer was found in mice immunized with pMd or pSMd. Co-injection of these mice with adjuvant had no effect on An production. Taken together, all the data above suggest that pIL-5 can enhance the antibody response in mice already had antigen specific antibody, but not in those without antigen-specific antibody.

IgG subtype is used as an indicator to determine the types of induced immune response. The IgG1 and IgG2a titers were first deermined to distinguish antibody response and cellular response (Fig.6). Generally, IgG1 titer was lower than IgG2a in both groups immunized with pSMdFc or pSMdFc in the presence of pIL-5. Moreover, IgG1 and IgG2a response were both higher in mice coinjected with pIL-5 was

co-injected. In groups of pSMdFc with pIL-5, mice showed significant IgG1 titer since the third immunization (6^{th} week) till 14^{th} week which suggested that mirine IL-5 plasmid as a potent genetic adjuvant in humoral immunity enhancement.

pIL-5 was co-injected to exaime whether antigen specific IgA can be enhanced. However, No significant enhancement in IgA production was observed (Fig.7).

We also investigate the optimal dose of plasmid inoculation. It showed that (Fig.8) mice immunized with 50μg pSMdFc alone or together with 50μg pIL-5 had similar IgG response when compared to those immunized with 100μg plasmid alone or with adjuvant. IgG titer of mice immunized with 25μg plasmid or adjuvant can also be detected, though the magnitude is less significant compared to other groups.

There is no neutralization ability of M2 antibody against A/PR/8/34:

Plaque size of A/PR/8/34 was not reduced while incubated with serum from immunized mice (Fig.9). Though the pfu is fewer than others and positive control when virus was pre-incubated with serum diluted 1:4 (Table 2).

DISCUSSION

In a previous study done in our lab, M2 specific antibody was successfully elicited in mice by inoculation of DNA vaccine expressing the full length M2 gene, but not by the transmembrane domain deleted forms, deletion of amino acids 26-43 or 25-55, of M2 gene. In order to elucidate the disability of truncated form M2 DNA vaccine, we further modified the truncated M2 gene deleted at the amino acids 25-55. Two modifications were made by adding a signal peptide of *staphylococcus aureous* protein A and/or human IgG1 Fc fragment were added to the N- and C- terminus of M2, respectively. The modified constructs, pSMd and pSMdFc were compared with pMd in the ability to induce the immune response in mice. However, the immune response was only induced in mice with inoculation of M2d with Fc fragment, either pSMdFc alone or with adjuvant pIL-5. The signal peptide fusion had no effect on immune induction as comparing to pM2d and naïve mice.

Theoretically, secreted protein is a better antigen to elicit immune response (10). In this study, we used intramuscular injection method to transfer our DNA vaccines where myocyte was the target cell to be transfected (27). Myocyte is not an immuno-competent cell due to lacking MHC class II and the associated molecules. However, myocyte is now recognized as an antigen-producing factory (6) serving as a long-term antigen source. Cecilia et al. (2) have successfully demonstrated the improvement in secretion of nef protein by fusing a murine heavy chain IgG signal peptide at N-terminus. Taken together, we determined to maximize M2 antigen secretion by fusion of a signal sequence to the N-terminus of M2d, so that it can be efficiently expressed and secreted from myocyte.

We determined to use the signal peptide of *Staphylococcus aureus* protein A. The fusion of this signal peptide to different proteins has demonstrated to be able to work successfully in a variety of expression systems, though most of which are prokaryotic

systems (15). Whether the signal peptidase in eukaryotes was capable to recognize the fused signal peptide from prokaryotes was checked by the SignalP V1.1 (9). The software predicted that the most possible cleavage site was at 39 (Ala) and 40 (Ser) which was at the junction of the signal peptide and the M2 gene, so there is no problem in cleavage of signal sequence of prokaryotes in eukaryotes cell.

We tried to study the effect of the signal peptide fusion to M2 antigen and found that there was no significant enhancement, neither in protein secretion in vitro nor in antibody response in vivo. There were two possible explanations for the failure to induce antibody response in group immunized pMd or pSMd. Firstly, the conformation of the truncated M2 protein made it less immunogenic as comparing to the wild type in B cell activation. Secondly, post-transcriptional or post-translational process, which might be related to the protein stability of pM2d and pSMd.

As for the first speculation, Frace et al. (18) have expressed similar M2 deletion proteins (deleted in residues 26-43 or residues 26-55) in E. coli and successfully elicited immune responses in mice immunized with these two recombinant proteins. Both M2 deletion proteins were found to be able to induce M2 specific antibodies and to enhance viral clearance in the lung of mice challenged with either homologous or heterologous influenza A viruses. The 14C2 epitope is maintained in these M2 deletion proteins expressed in E.coli. It seems that all the post-translational modifications are not required to maintain the antigenicity. Therefore, we suppose that the conformation of the M2 deletion proteins expressed in vivo possesses similar effect on immune activation.

Since there is no impediment in the M2 deletion gene expression in mammalian system, the stability of M2 deleted protein is concerned. The transmembrane domain of M2 protein is critical in formation of an integral membrane protein. This region has a hydropathic index of >20 as calculated by Kyte and Doolittle method (65). There

are a few evidences supported the importance of transmembrane domain in protein stability. Chun et al. (4) have studied the membrane domain in the regulated degradation of 3-hydroxy-3-methylglutaryl coenzyme A reductase and found that the deletion of 64 amino acids from the central region of the transmembrane domain causes the protein to be degraded extremely rapidly. The phenomenon has also been found in influenza A virus. Doyle et al. (7) have found that the progressive deletions in transmembrane domain of hemagglutinin, another transmembrane protein in influenza virus, resulted in drastic alternations in transport, membrane association, and protein stability. Taken together, we suspected that the truncation of M2 transmembrane domain might influence the stability of M2 protein in cytosol.

Moreover, Gred et al. (8) have found that the region at 15-20 residue segment C-terminal to the M2 transmembrane domain was highly resistant to digestion by chymotrypsin and trypsin and suggested that this region might help to stabilize the tetrameric M2 protein formation. 15-20 residue segment C-terminal to transmembrane domain is partially truncated in our construct M2d(25-55) where 15 residue segment C terminal to transmembrane domain, and as the result, may sensitized the M2 deletion protein to a variety of proteases in cytosol and lead to much shorter half life of M2 protein. Moreover, cysteine residue 50 was found to be a palmitoylation site for fatty acid addition by thio-ether linker (16). In the case of mannose 6-phosphate receptors (MPR), Breuer et al. (21) have found that the spacing between palmitoylation site and transmembrane domain is important for a post Golgi sorting step preventing receptor degradation. Besides, Christina et al (3) have observed that palmitoylation of the Rous Sarcoma Virus transmembrane glycoprotein is required for protein stability where the mutated in the palmytolation site in transmembrane domain leads to rapidly degradation. Furthermore, the increased rate of internalization into endosomes and lysosomes is related to the paralleled decrease in palmitoylation.

Taken together, palmitoyl group is not only critical in association with lipid membrane but also for protein stability.

The length of the linker is also speculated to be responsible to the stability of deleted M2 protien in the cytoplasm. Prescott (17) studied the effect of the length of the polypeptide linker in the stability of the fusion protein. He found that the longer the linker is, the lower the degradation of fusion protein is. The linker was used to fuse the extracellular domain and the cytoplasmic domain to get a M2 deletion (25-55). The polypeptide used here contained only four amino acids (G_4S_1) which was shorter than those commonly used. It takes further investigation whether the stability of M2 d(25-55) is influenced by the length of the linker we used here.

Antigen presenting cells are the major immuno-competent cells, which can display peptides associated with MHC class II molecule to $CD4^+$ T cells. It also displays a variety of Fc receptors for immunoglobulin. In order to enhance the processing and presenting of M2 antigen by antigen presenting cells, a human IgG1 Fc fragment was added to M2d. Haeffner et al. (19) have demonstrated the binding affinity of Fc fragment of human IgG1 and murine Fc receptor. He found that the binding affinity of human IgG subclasses to P388D1 cells, a murine macrophage cell line shows a hierarchy: IgG3>IgG1>IgG4>>IgG2. Therefore, the usage of human IgG1 Fc as the fusion partner to target M2 to the Fc receptor on antigen presenting cells might be practicable. Firstly, we tried to demonstrate the binding affinity of Fc-fusion protein in vitro. We expressed a recombinant protein, M2dFc, by E.coli. pMAL is the expression vector which contains a maltose binding protein at the N-terminus to facilitate the purification of recombinant protein. The signal peptide was removed to mimic the protein expressed in vivo. MBP-M2dFc was used to interact with P388D1 at 0□ and 37□ to study the binding and internalization of ligand, respectively. The result was not very significant when compared to the control,

MBP-M2d. Clark et al. (5) have characterized the Fc receptor of IgG on U937, a human macrophage cell line, and found that the specific binding of IgG1 to its Fc receptor is only 17.3 %. It is not high enough to distinguish from the non-specific binding of Fc receptor, which is approximately 7% (1).

Besides targeting of M2 to antigen presenting cells via Fc receptor, Fc fusion might provide another function for M2d. The Fc fragment was from the nt785-nt1468 of human IgG1 gene, which contained the hinge region to CH3 domain. As described previously, we speculated that the failure of pMd and pSMd to elicit an immune response might be due to the protein stability of truncated structure in vivo. However, pSMdFc could successfully induce an antibody response. In addition to the fusion of Fc fragment to M2d in directing the antigen for efficient processing and presentation, the hinge region also played a pivotal role in SMdFc protein stability. It was not only providing a disulfide link between Fc fragments but also serving as a protease resistant region. Kim et al. (13) studied the hinge region of IgG1 and found that the hinge region plays a role in maintaining serum levels of the murine IgG1 by lowering the susceptibility to pepsin attack. Therefore, we speculated that the fusion of M2d to hIgG1 Fc increased the stability of the fusion protein and prolonged the half-life for the antigen to be accessible to the antigen presenting cells.

The antibody response was successfully induced in mice inoculated with pSMdFc alone or with pIL-5, and the antibody titer was comparable to those inoculated with plasmid containing the full length M2 gene. The antibody titer of mice immunized with pSMdFc alone or with pIL-5 at the 8^{th} week were 2^{12} and $2^{12.15}$, respectively, while the antibody titer of mice immunized with plasmids containing no Fc fragment gene were lower than 2^5. IgG1 was lower than IgG2a either in mice immunized with pSMdFc alone or with pIL-5. IgG1 is an indicator of humoral response, while IgG2a is an indicator of cellular response. Generally speaking, CTL

response is mainly induced by intramuscular injection of DNA vaccine where IgG2a is augmented in this response. According to a higher IgG2a content in our result, we showed CTL response was elicited after intramuscular injection of pSMdFc. Furthermore, pIL-5, a Th2 cytokine, induced both humoral and cellular responses at 8^{th} and 10^{th} (IgG1/IgG2a=0.8-1), while compared to the mice immunized with pSMdFc alone (IgG1/IgG2a=0.2-0.4). Though effect of pIL-5 does not last, it did prolong the duration and augment the magnitude of IgG response against M2. The titer of pSMdFc+pIL-5 at 14^{th} was $2^{11.3}$ (data not shown). Furthermore, in dose response experiment, we found that as low as 25μg of pSMdFc alone can induce a significant antibody titer, though it does not induce as a high titer as 50μg or 100μg of pSMdFc. The IgA was not enhanced after inoculation of pIL-5. IL-5 can stimulate the differentiation of IgA^+ B cells which dominantly exist in the mucosal area rather than muscle. We used intramuscular route to deliver the DNA plasmid instead of intranasal route, which may reduce the local effect of the IL-5. The intranasally inoculation of the DNA plasmids is under investigation to study the optimal route for IL-5 as an adjuvant for IgA enhancement. Others investigated CTL response as well in our lab and found that CTL response was induced in mice immunized with pMd but failed to detect antibody response by administration of pMd. Sang et al. (22) found that the threshold for the induction of CTL response is lower than that of IgG response, which may account for the failure of detection of the antibody response in our study.

The antibody, 14C2, of M2 showed the ability to restrict the virus growth by reduction of the plaque size after addiction of M2 antibody, though only found in A/Udron/72, A/Singapore/1/57, A/FW/1/50, A/USSR/90/77, and A/HK/8/68, but not in A/WSN/33 and A/PR/8/34 (24). It was noteworthy that there were no amino acids different in the extracellular N terminal of these strains, whereas, few changes were found differ from those in M2 antibody-sensitive strains. Residues 28 and 31 at

membrane-spanning region and residues 54, 57, 89 at cytoplasmic domain (23) showed differed in antibody-sensitive strains. It is possible that M2 specific antibody can affect influenza virus assembly by inducing a clustering of M2 on viral particle or infected cells (20). The antibody binding could induce a conformational change in the M2 protein and affect virulence of most antibody-sensitive influenza A virus. In our neutralization test, A/PR/8/34 was used as the virus to study the protection ability of the antibody induced by pSMdFc. It might not be suitable to use an antibody-resistant strain of influenza A virus and some antibody-sensitive strains might be required to address this question. Either antibody sensitive strains (i.e. A/HK/8/68) or antibody resistant strains (i.e. A/PR/8/34) are usually used (11, 12, 18, 25). All of them show that a prevalent protection from viral challenge. Walter et al. (26) have studied the role of B-cell response in recovery of mice from primary influenza virus infection, and found that anti-M2 antibody suppressed the virus titer in the lung but failed to clear the infection which is due to the inability of neutralization ability of M2 specific antibody. Even though, virus opsonized by M2 specific antibody may become neutralized through uptake by Fc receptor expressing or complement expressing cells. In conclusion, M2 specific antibody induced by either recombinant proteins or DNA vaccines might protect the mice from both antibody-sensitive and antibody-resistant strains challenge through either neutralization activity or the opsonization activity of the virus.

We have demonstrated that using Fc fragment to target antigen to antigen-presenting cells can induce or enhance the immune response in antigens which were less potent. This might be a convenient way to apply in other antigens as well.

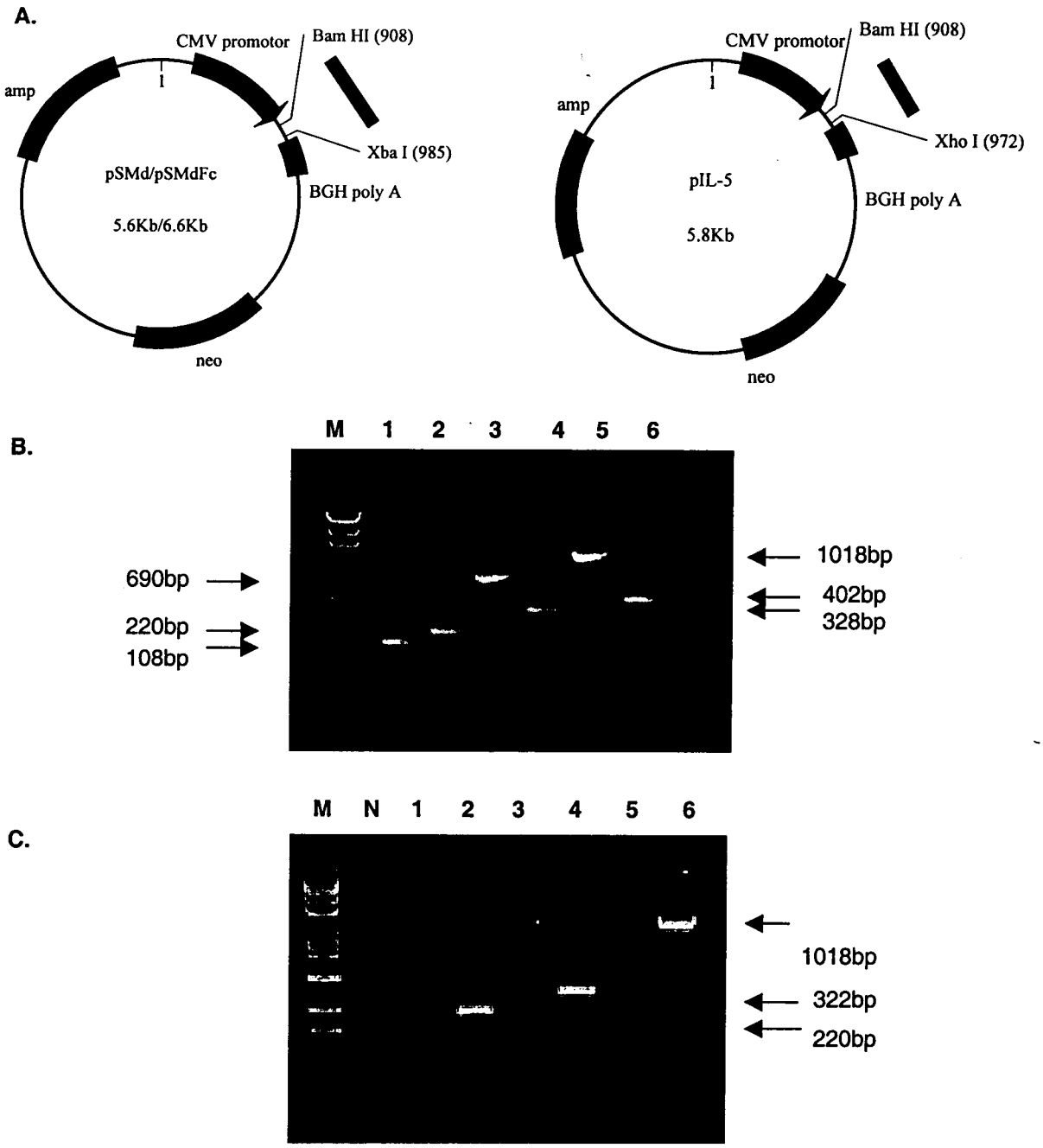


Fig.1 Genetic maps and gene expressionby PCR and RT-PCR. (A) SMd and SMdFc were inserted into the pcDNS3 plasmid vector between BamH I site and Xba I site, while murine IL-5 was inserted between BamH I site and Xho I site. CMV promoter: the immediate early gene promoter of human cytomegalovirus. BGH poly A: derived from bovine growth hormone gene. amp: ampicillin. Neo: neomycin. (B) Signal peptide, M2 d(25-55), and hIgG Fc were amplified and served as templates to get SMd and SMdFc by secondary and third PCR, respectively. Lane M: 1Kb DNA ladder. Lane 1: signal peptide of *Staphylococcus aureous* protein A, 108 bp. Lane 2: M2 d(25-55), 220 bp. Lane 3: human IgG1 Fc, 690 bp. Lane 4: SMd, 328 bp. Lane 5: SMdFc, 1018 bp. Lane 6: murine IL-5, 402 bp. (C)RNA was extracted from 293T cells transfected with M2 plasmids; pMd, pSMd, and pSMdFc. The RNA was divided into two parts, each of which was prepared for PCR (1, 3, 5) or RT-PCR (2, 4, 6). M: marker, N: negtive control. 1, 2: pMd. 3, 4: pSMd. 5, 6:pSMdFc.

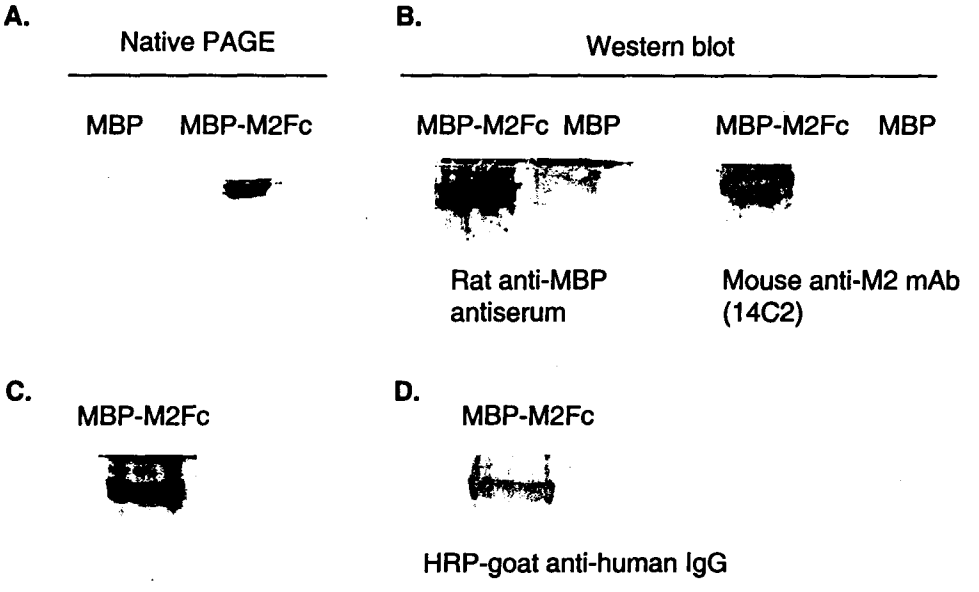


Fig.3 Native-PAGE and western blot of MBP and MBP-M2Fc. The conformation of MBP and MBP-M2Fc were confirmed by western blot after running in native-PAGE. Though MBP was not visualized in native-PAGE, it was recognized by its antibody in western blot. A, C: native PAGE of MBP and MBP-M2Fc. B, D: western blot of MBP and MBP-M2Fc by first antibody; rat anti-MBP-antiserum, mouse monoclonal antibody 14C2, and HRP-goat anti-human IgG for MBP, M2, and human IgG Fc detection.

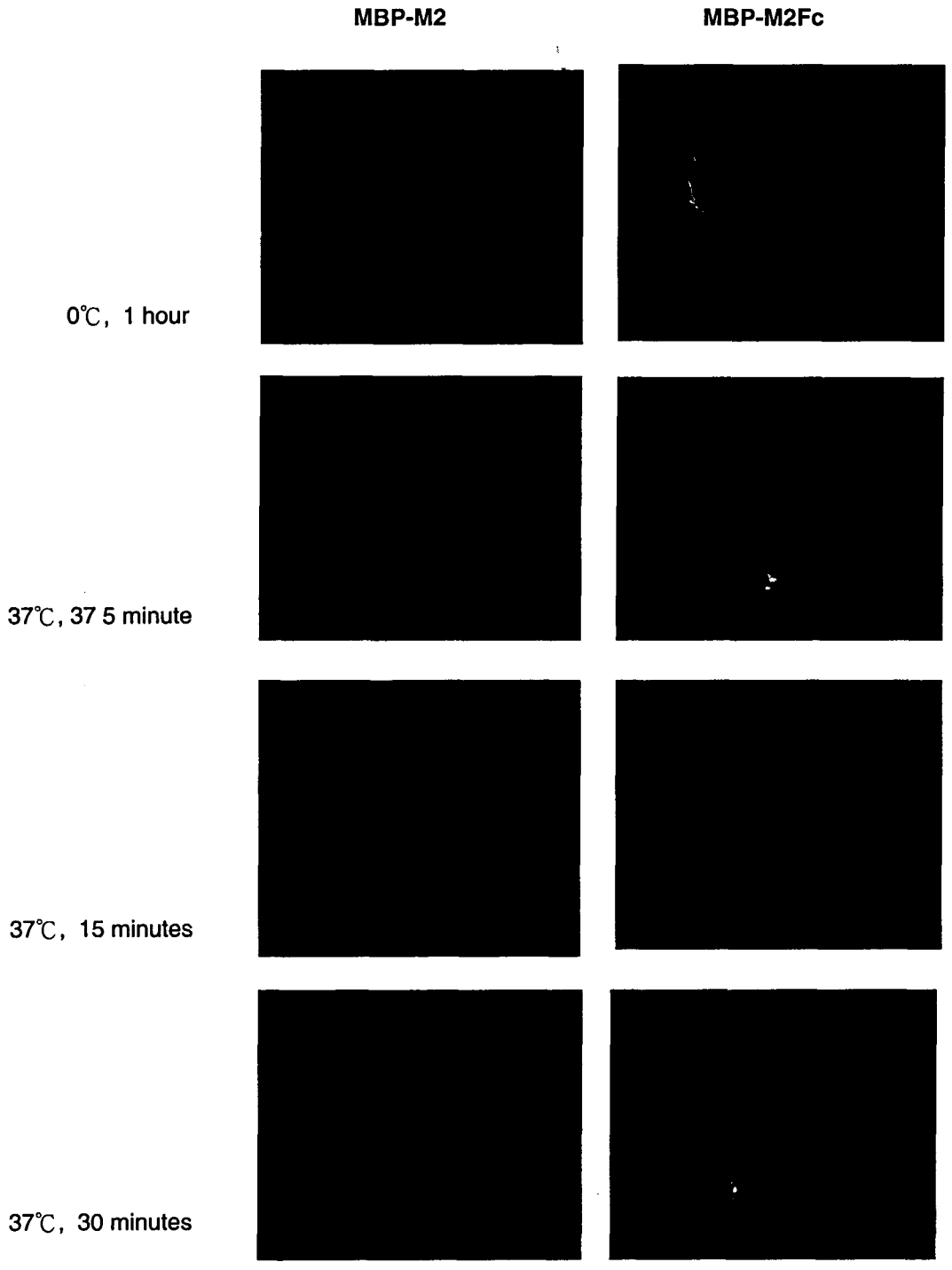
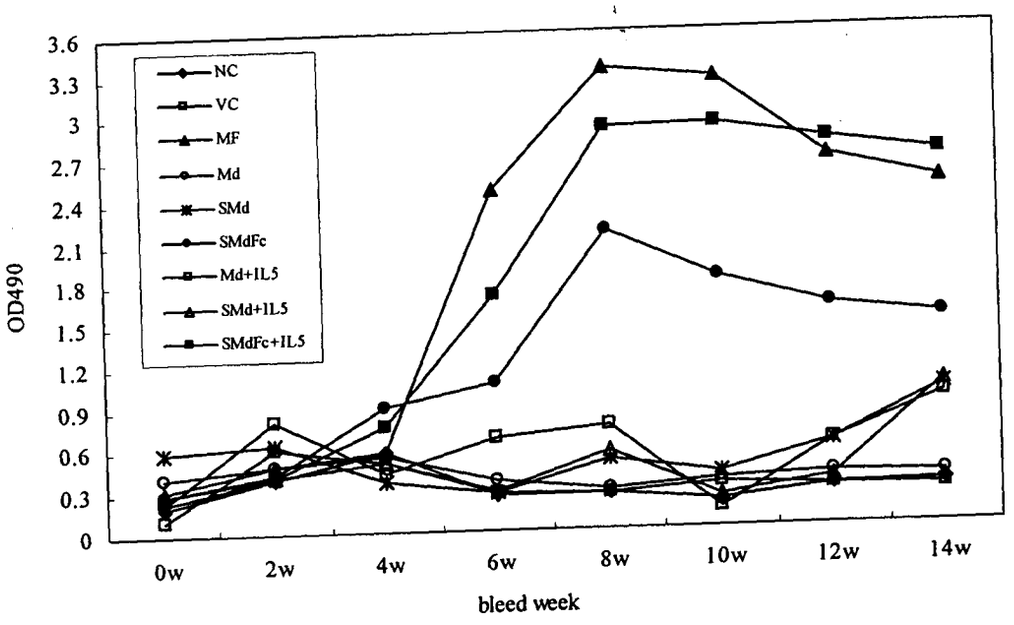


Fig.4 Fc-mediated targeting and endosytosis of M2d. M2d was targeted to P388D1 via the interaction between the Fc fragment and the Fc receptor on cell surface. Cells were counter stained with Evaus blue, and mouse monoclonal antibody 14C2 was used as the first antibody and M2 was visualized by FITC-goat anti-mouse IgG. Fc mediated endocytosis was visualized after transferring to 37℃ and receptor complex was digested within 30 minutes.

A.



B.

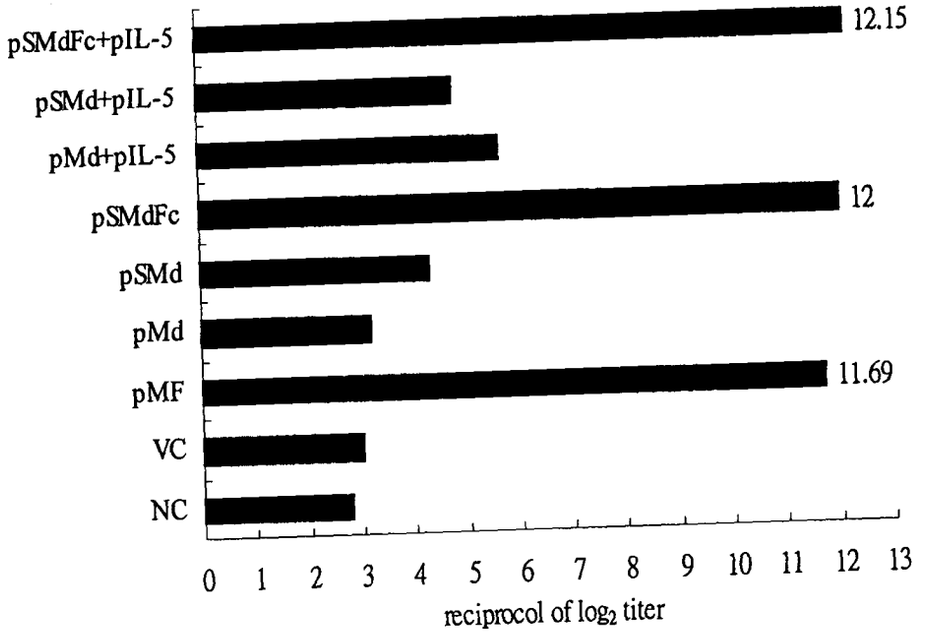


Fig.5 IgG antibody and antibody titer of immunized mice against influenza A virus M2 protein. (A) Measurement of IgG Antibody response in mice that received three doses of 100 μg M2d DNA with different modifications with two weeks interval via intramuscular injection. Serum samples were collected before immunization and biweekly until the 14th weeks. Serum was diluted 1:40 before the antibody measurement. (B) IgG antibody titer was expressed as the reciprocal of the final detectable dilution values, which have an OD490 > three folds of standard deviation of negative control.

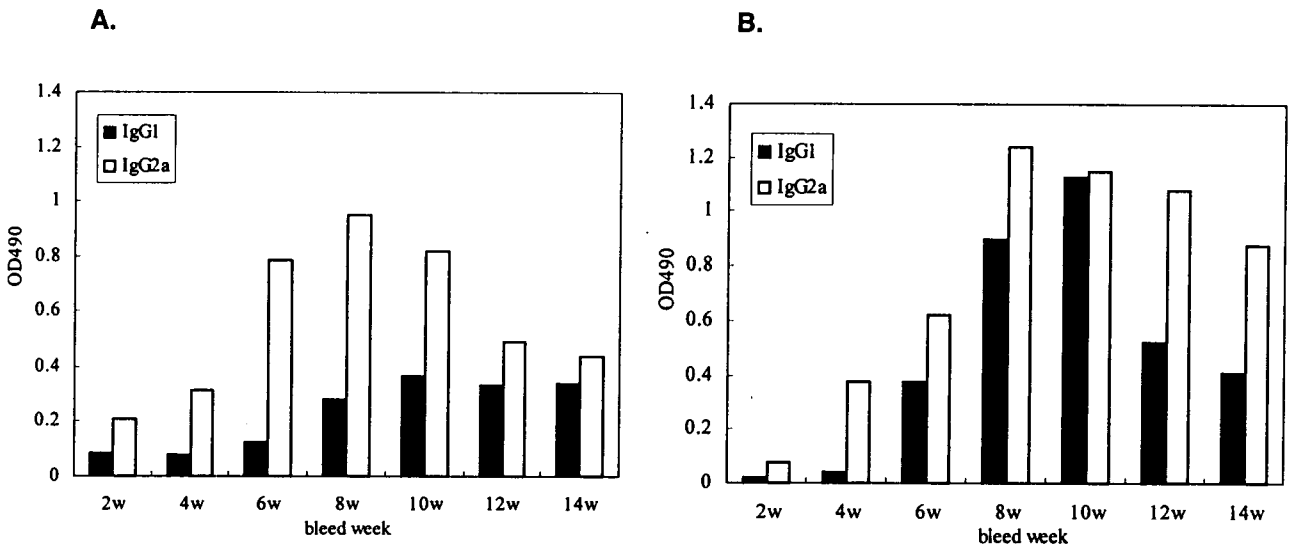


Fig.6 Measurement of IgG antibidy subclass titer of immunized mice against influenza A virus M2 protein. Serum from each group was collected and IgG1 and IgG2a antibody were measured by HRP-goat anti-mouse IgG1 and HRP-goat anti-mouseIgG2a, respectively. Serum was diluted 1:40 before antibody measurement. A: IgG1 and IgG2a of mice immunized with pSMdFc. B: IgG1 and IgG2a of mice immunized with pSMdFc+pIL-5.

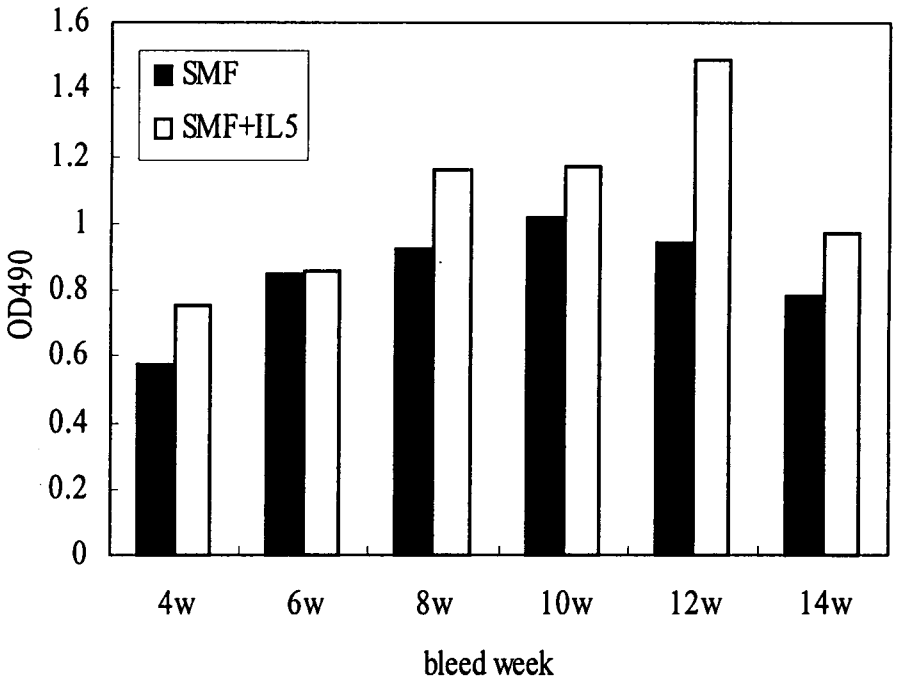


Fig.7 Measurement of IgA antibody of immunized mice. IgA was detected by HRP-goat anti-mouse IgA and serum was diluted 1:10 before antibody measurement.

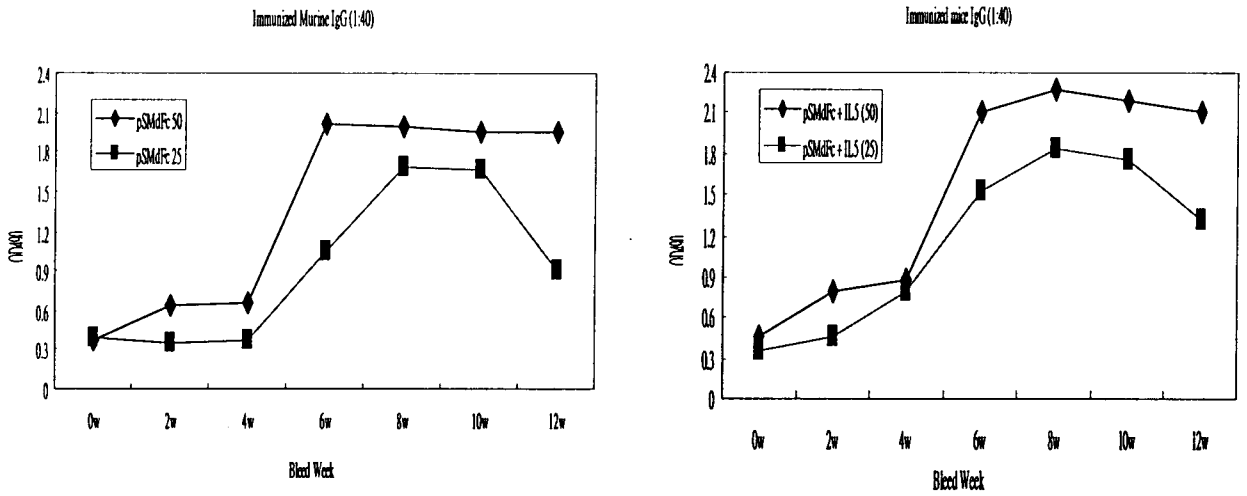


Fig.8 IgG antibody of mice immunzied with different doses of plasmid. Measurement of IgG Antibody response in mice that received three doses of 50 μg or 25 μg pSM2dFc DNA with different modifications with two weeks interval via intramuscular injection. Serum samples were collected before immunization and biweekly until the 8th weeks. Serum was diluted 1:40 before the antibody measurement.

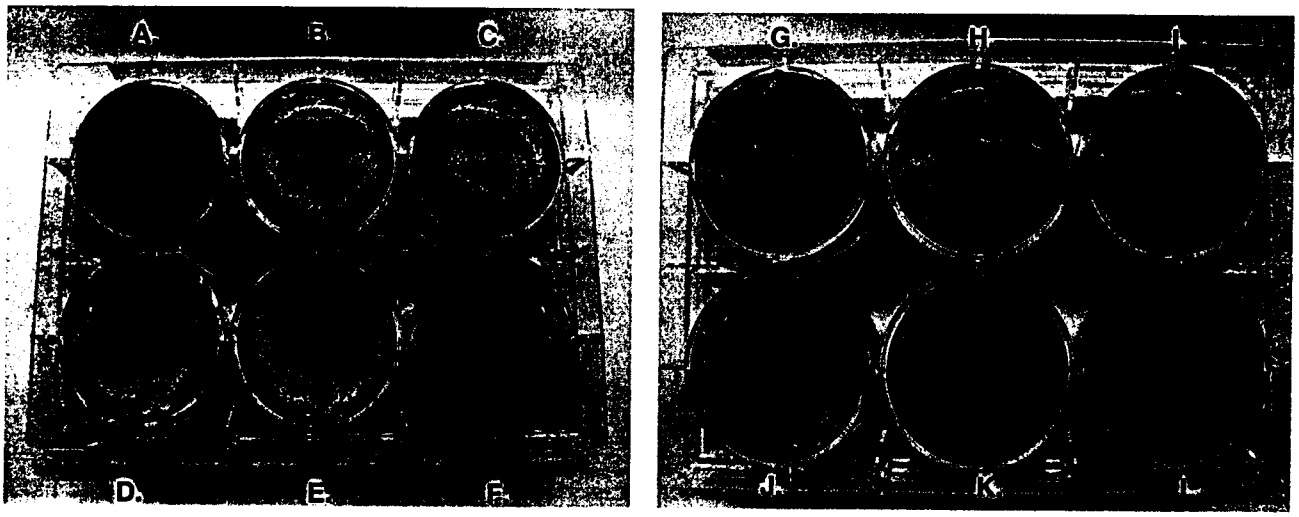


Fig.9 Neutralizing test of antibody against M2. Serum from mice immunized with pSMdFc+pIL-5 were pooled and diluted 1:4 to 1:32 for neutralization test. A/PR/8/34 was used to infect MDCK cells, and 14C2 was used as the positive control. A: serum control. B, E: virus control. C: cell control. D. 14C2 control. F. 32x. G,J: 4x. H, K: 8x. I, L: 16x.

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計畫成果自評；

本計畫完成 M2 基因的擇殖、修飾，並已成功的表現蛋白質，可以提供後續實驗抗體偵測之用。小鼠之免疫實驗顯示 DNA 疫苗確實可激發抗體反應，但較之蛋白質之免疫反應為弱，如何增進其免疫反應，且增強 IgA 抗體反應，是需要努力的方向。在 M2 蛋白質剔除穿膜部分之基因之前或後連結 Fc 片段或訊號生太序列，有助於增強免疫反應。此研究成果具有應用之價值。