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Immunobiology

Recombinant protein comprising multi-neutralizing epitopes induced high titer of antibodies against Influenza A virus

Hua Li, Jian Ding, Ying-Hua Chen

Laboratory of Immunology, Research Centre for Medical Science and Department of Biology, Tsinghua University; Protein Science Laboratory of MOE, Beijing 100084, P. R. China

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Abstract

In previous studies, we suggested that epitope-vaccine might be a new strategy against virus infection. Based on this hypothesis, we designed and expressed a recombinant immunogen (multi-epitope-peptide) comprising repeats of three neutralizing-epitopes (neutralizing epitopes: aa92–105, 127–133 and 183–195) of hemagglutininin (HA) of influenza virus (H3N2) in *E. coli*. After vaccination, the recombinant multi-epitope protein could induce a high level of antibodies with predefined multi-epitope-specificity in mice and rabbits. The epitope-specific antibodies in sera were tested using three different epitope-peptides (synthetic peptides) in ELISA assay, and the serum dilutions from 1:6400 to 1:25600 were confirmed. In western blot analysis, both the antiserum and the antibodies purified by synthetic epitope-peptide coupled sepharose columns could recognize natural HA from influenza virus particles (strain A/Wuhan/359/95 H3N2). In hemagglutination inhibition (HI) tests, these three antisera at the dilutions from 1:20 to 1:80 showed inhibitory activity. Interestingly, antisera and purified antibodies induced by the epitope-vaccine could partially inhibit plaque-formation of influenza virus (strain A/Wuhan/359/95) on MDCK cell monolayers. These results suggest that the recombinant multi-epitope vaccine can simultaneously induce multi-antiviral activities against influenza virus, which may provide a new way to develop effective vaccines against influenza virus.

Introduction

Influenza is a mild febrile illness in general, but it causes severe symptoms and sometimes death in those who have underlying diseases or in the aged. Large influenza epidemics, even at the present time, become serious public health problems all over the world. It has been generally considered that neutralizing antibodies are cardinal for protection against influenza A infection (Virelizier, 1975; Ada & Jones, 1986). However, the effect of currently available vaccines against influenza is extremely variable (Cox & Subbarao, 1999). To solve the problem, intranasal immunization (Corrigan & Clancy, 1999), new adjuvants (Martin, 1997) and increasing the dose of hemagglutinin (HA) and neuraminidase (NA) antigens with purified protein have been used (Couch et al., 1997). In previous studies, we suggested epitope-vaccine as a new strategy to induce multi-antiviral activities (Chen et al., 1999; Xiao et al., 2001).

Corresponding author: Prof. Dr. Ying-Hua Chen, Laboratory of Immunology, Research Centre for Medical Science and Department of Biology, Tsinghua University; Protein Science Laboratory of MOE, Beijing 100084, P. R. China. Phone/Fax.: +86-10-6277 2267; E-mail: chenyh@mail.tsinghua.edu.cn

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The membrane protein HA of influenza A viruses, mediating the attachment of the virus to target cells and the release of viral content into attached cells, is the major target of neutralizing antibody responses (Wilson & Cox, 1990; Skehel & Wiley, 2002). After decades of research, several neutralizing epitopes on HA have been identified. The peptide (HA aa127–133) could induce neutralizing antibodies and T cell response after introduction into an MHC II binding component (Naruse et al., 1994; Ogasawara et al., 1992). The peptide (HA aa183–199), when conjugated to tetanus toxin (TT), can induce both humoral and T cell responses and protect Balb/c mice from lethal virus infection (Simeckova-Rosenberg et al., 1995). The peptide (HA aa91–108) can also elicit immune responses and lead to partial protection (Muller et al., 1982; Shapira et al., 1984). Intersubunit region of HA (aa333–357) is immunogenic in Balb/c mice without the administration of any carrier (Rajnavolgyi, 1992). Hybrid flagella containing one B, T_H and CTL (cytotoxic T lymphocyte) epitope of influenza virus showed significant protection against sub-lethal viral challenge (Simeckova-Rosenberg et al., 1995; Jeon et al., 2002).

Although epitope-vaccines prepared by synthetic epitope-peptides have shown some distinct properties and advantages in comparison with the traditional vaccine (Lu et al., 2002), it is necessary to develop a new method together with synthetic-peptide-based epitope-vaccine to fulfill the multi-epitope-vaccine strategy. In this study, we designed and expressed a recombinant immunogen containing three neutralizing epitopes on HA1 (aa92–105, 127–133, 183–195) of influenza virus (H3N2), and wanted to induce high levels of antibodies with predefined multi-epitope-specificity against influenza virus.

Materials and methods

Peptides and antibodies

Three epitope-peptides (P1, P2 and P3) containing neutralizing epitopes on HA of influenza A virus (strain A/Wuhan/359/95, H3N2) and a control peptide (CP) were commercially synthesised in Genemed Synthesis Inc. (USA). P1: C-(KAYSNCYPYDVPDYG)$_2$, HA aa92–105; P2: C-(GHHPITDSDQTRLY)$_2$, HA aa183–195; P3: C-(GWTGVAQD)$_2$, HA aa127–133; CP: $(KGGG)_7$-K. Peroxidase-conjugated rabbit anti-mouse and goat anti-rabbit immunoglobulin were obtained from Dako (Denmark); mAb His · Tag was obtained from Novagen (USA); control mAb 10E3 was prepared in our laboratory (Tian et al., 2001). Three epitope-specific antibodies against P1, P2 and P3 were purified from the rabbit antisera against the peptides P1, P2 and P3 using peptide-coupled NHS-activated sepharose columns.

Construction and expression of recombinant immunogen gene

Based on the nucleotide sequence of influenza virus A/Wuhan/359/95, three oligonucleotide fragments encoding repeats of HA peptides aa92–105, 183–195 and 127–133 respectively, and five primers were synthesized (Sangon, China) (Table 1). Primers F1/R1 were used to amplify the O1 fragment while primers F2/R2 were used to amplify the O2 fragment. 100 ng of each product were mixed together and used as templates for overlap extension PCR using primers F1/R2. After two steps of PCR, four restriction enzyme sites (*Bam*HI, *Sal*I, *Bgl*II, *Xho*I) were introduced into the linked DNA fragment O1–O2. The fragment was inserted into pGEM T-easy vector (Promega, USA) and repeated once using *Bam*HI, *Bgl*II and *Xho*I. The primers F3/R2 were used to amplify the fragment O3. A fragment of four repeats of O3 was generated as mentioned above and linked downstream to the fragment $(O1-O2)_2$. The target gene sequence was determined by the didoxynucleotide chain termination method.

After cutting from the recombinant T-easy vector using *Bam*HI/*Xho*I, the multi-epitope gene was ligated into similarly treated expression vector pET-28a (Invitrogen, USA) to yield recombinant plasmid pET-epitope. Competent *E. coli* strain BL21 cells were then transformed and grown in 2 × YT agar medium containing 60 μg/ml kanamycin sulfate. Overnight cultures of the recombinant bacteria having plasmid pET-epitope were diluted 1:50 in 200 ml 2 × YT mediums and grown at 37 °C for about two hours. 1 mM isopropylthiogalactoside (IPTG) was added to induce protein expression. After a further 4 hours incubation under vigorous agitation, *E. coli* cells were pelleted for further purification.

Purification and identification of recombinant protein (immunogen)

The cell paste was resuspended in 20 ml ice-cold PBS and sonicated twice for 2 minutes with 10 minutes rest on ice. The pellet was collected by centrifugation at 7800*g*, resuspended in PBS containing 0.1% Triton X-100, sonicated briefly, and centrifuged at

Table 1. Oligonucleotides and primers used for generation of the multi-epitope antigen gene containing three different neutralizing epitopes. Primers F_1/R_1 or F_2/R_2 were used for amplifying DNA fragments O_1 and O_2 corresponding to $(KAYSNCYPYDVPDYG)_2$ and $(GHHPITDSDQTRLY)_2$ separately. Primers F_3 and R_2 were used to amplify DNA fragment O_3 corresponding to $(GWTGVAQD)_2$

O1	5'-GGA TCC AAA GCT TAC AGC AAC TGT TAC CCT TAT GAT GTG CCG GAT TAT GGC AAA GCT TAC AGC AAC TGT TAC CCT TAT GAT GTG CCG GAT TAT GTC GAC CAC CAC CCG-3'
F1	5'-GTG GAT CCA AAG CTT AC-3'
R1	5'-CGG GTG GTG GTC GAC-3'
O2	5'-TAT GTC GAC CAC CAC CCG AGT ACG GAC AGT GAC CAA ACC AGC ATA TAT GGC CAC CAC CCG AGT ACG GAC AGT GAC CAA ACC AGC ATA TAT AGA TCT TGA GCT CGA GTC-3'
F2	5'-TAT GTC GAC CAC CAC CCG-3'
R2	5'-CGA CTC GAG CTC AAG ATC T-3'
O3	5'-GGA TCC TGG ACC GGA GTG GCG CAG GAC GGC TGG ACC GGA GTG GCG CAG GAC GGC TGG ACC GGA GTG GCG CAG GAC AGA TCT TGA GCT CGA G TCG-3'
F3	5'-GCG GAT CCT GGA CCG GA-3'

2000*g*. The inclusion bodies were then solubilized in 50 mM sodium phosphate buffer (PBS), pH 7.5, containing 0.5 M sodium chloride, 5 mM imidazole, and 6 M urea (buffer A). Insoluble material was removed by centrifugation at 17,000*g*. The supernatant was loaded onto a Ni^{2+}-NTA column pre-equilibrated with buffer A. After further equilibration, recombinant protein was eluted with the same buffer containing 250 mM imidazole. The refolding procedure on the Ni^{2+}-NTA column was used. After dialyzing in buffer A, purified protein was reloaded onto the column and refolded by applying a 1000 ml reverse gradient of urea (6 to 0 M) in the same buffer at a flow rate of 2 ml/minute at room temperature. The column was then washed with 80 ml of 50 mM PBS, pH 7.5, containing 0.5 M sodium chloride, 5 mM imidazole. Refolded protein was eluted in the same buffer with 250 mM imidazole. After dialyzing with 20 mM Tris-HCl, pH 7.8, the purified recombinant fusion protein was tested by western blot analysis as described in Ding et al. (2000).

Immunization and identification of epitope-specific antibodies in ELISA

After mixing with complete Freund's adjuvant, each of four mice was immunized with 10 μg recombinant protein intraperitoneally while each of three rabbits was immunized with 50 μg antigen subcutaneously at axillea and groin. Boosters with the same dose were given in incomplete Freund's adjuvant on days 11, 22 and/or 33. Antisera were separated on the 5th day after the last booster. Pre-immune sera were collected before immunization. Epitope-specific antibodies were detected in the enzyme linked immunosorbent assay (ELISA) as described in Tian et al. (2001).

Isolation and identification of epitope-specific antibodies

Antiserum of rabbit was loaded onto peptide-coupled sepharose-4B. After equilibration with PBS (pH 7.5), epitope specific antibodies were eluted with 0.1 M glycine (pH 2.7). Influenza virus particles (A/Wuhan/359/95) were subjected to electrophoresis and electroblotted onto NC membrane. Binding of epitope specific antibodies to HA were detected by western blot analysis as described in Ding et al. (2000).

Hemagglutination inhibition assay (HI assay)

Hemagglutination unit (HU) of the influenza virus was titrated using chicken red blood cells (RBCs). Allantoic fluid was serially diluted with PBS (pH 7.2) in V-shaped 96-well microtiter plates, 25 μl in each well. Another 25 μl PBS (pH 7.2) and 50 μl 0.5% chicken RBCs suspension in PBS were added to each well separately. A 4 HU/25 μl virus suspension was prepared according to the results of the HA titration and back titrated. Sera were treated with trypsin and $NaIO_4$ to remove nonspecific agglutination inhibitors and nonspecific agglutinins. 25 μl of serially diluted serum was mixed with 25 μl

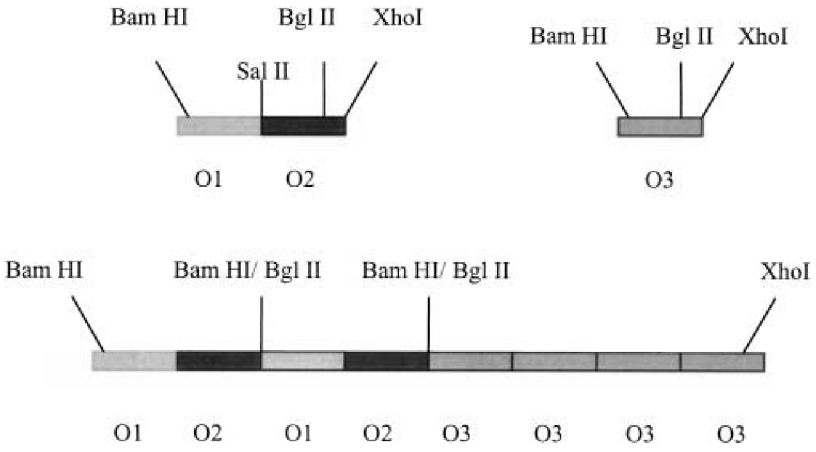

Fig. 1. Scheme of the construction of recombinant multi-epitope immunogen gene. O1, oligonucleotides corresponding to P1: C-(KAYSNCYPYDVPDY*G*)$_2$ (HA aa92–105); O2, oligonucleotides corresponding to P2: C-(*G*HHPITDSDQTRLY)$_2$ (HA aa183–195); O3, oligonucleotides corresponding to P3: C-(*G*WTGVAQD)$_2$ (HA aa127–133).

of virus suspension and incubated for 1 hour at room temperature. 50 µl 0.5% chicken RBCs in PBS (pH 7.2) was added. The HI endpoint was taken as the highest dilution clearly demonstrating hemagglutination of chicken RBCs.

Inhibition of plaque formation

Madin-Darby canine kidney (MDCK) cells (ATCC) were cultured to monolayers in 24-well plates (Costar, USA). Influenza virus A/Wuhan/359/95, grown in the allantoic cavity of 9-day-old embryonated eggs, was serially diluted in DMEM (high glucose) (Gibco, USA) ($4^0 \times 10^{-3}$ to $4^{-5} \times 10^{-3}$). After one hour incubation at 34 °C, the mixtures (100 µl per well) along with fresh medium (400 µl per well) were added to the plate and incubated for 2 hours at 34 °C. After removal of the medium, 1% agarose in DMEM was laid into each well. Non-serum DMEM was added after the agarose solidified. The plate was stained by 1% crystal violet after 72 hours incubation at 34 °C. Plaque number was counted and plaque formation unit (PFU) in the viral stock was calculated. Plaque formation inhibition was performed by the same procedure. Serial dilutions of antisera or antibodies were mixed with the same volume of medium containing 60 PFU of virus.

Results

In this study, a gene of a recombinant multi-epitope peptide (immunogen) bearing three epitopes on influenza HA protein was designed and constructed. The O1 and O2 fragments were joined together (O1 – O2) by using overlap-extension PCR, and then the O1 – O2 fragment was ligated into T-easy vector. Employing the enzymes of *Bam*HI, *Bgl*II and *Xho*I, the O1 – O2 fragment was repeated again and the O3 fragment was repeated four times after the repeats of O1 – O2. The final composition of the gene fragment becomes $(O1-O2)_2-(O3)_4$ (Fig. 1). The sequence analysis of the recombinant DNA demonstrated that no alteration had occurred during the synthesis process, PCR amplification or cloning. After sub-cloning into plasmid pET-28a and transformation into competent BL21 cells, the gene of the recombinant protein antigen was expressed in *E. coli* when induced with IPTG. Purified by Ni^{2+}-NTA affinity column, the recombinant protein (34 kDa) containing His · Tag and three different epitopes of HA were identified in western blot assay using mAb His · Tag (Fig. 2, lane B). Three epitope-specific antibodies purified from the rabbit antisera against the HA peptides P1, P2 and P3 could recognize the recombinant protein (34 kDa) respectively (Fig. 2, lanes D, E and F), while the control antibody (negative control) did not (Fig. 2, lane C). These results indicate that the recombinant protein (34 kDa) contains His · Tag and three different epitopes of HA.

After vaccination, the recombinant multi-epitope protein could induce a high level of antibodies with predefined multi-epitope-specificity in mice and rabbits. The epitope-specific antibodies in antisera were tested using three different epitope-peptides (synthetic peptides P1, P2 and P3) in ELISA assay, mouse antisera dilution up to 1 : 6400 – 1 : 25600 for P1, 1 : 6400 for P2, while P3-specific antibodies were

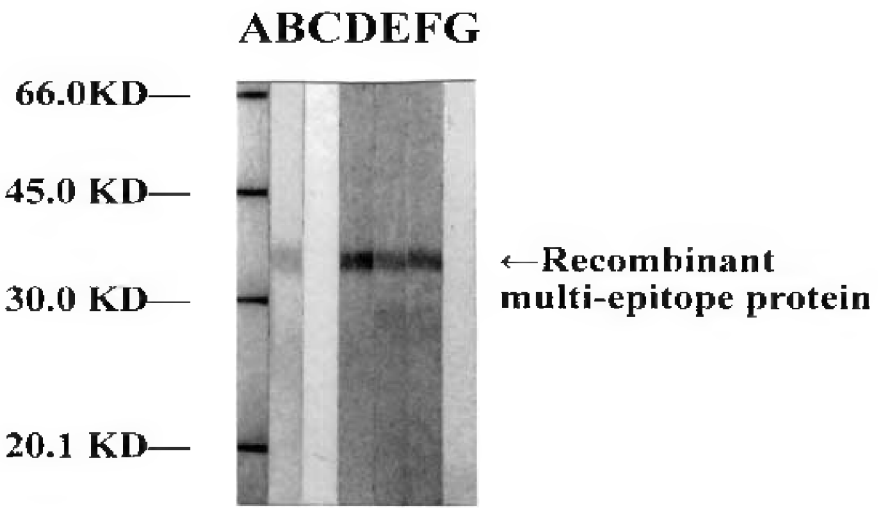

Fig. 2. Identification of the purified recombinant protein in western blot analysis. The purified recombinant multi-epitope proteins (20 μg) were subjected to electrophoresis in SDS-PAGE and electro-blotted onto nitro-cellulose membrane. The binding of specific antibodies to the recombinant protein was detected with peroxidase conjugated rabbit anti-mouse immunoglobulin and goat anti-rabbit immunoglobulin. Lane A, molecular weight marker; Lane B, binding of mouse mAb (His·Tag) to recombinant protein; Lane C, binding of control antibody (mouse antibody as negative control) to recombinant protein; Lane D, binding of peptide C-(KAYSNCYPYDVPDY*G*)$_2$ specific rabbit antibodies to recombinant protein; Lane E, binding of peptide C-(*G*HHPITDSDQTRLY)$_2$ specific rabbit antibodies to recombinant protein; Lane F, binding of peptide C-(*G*WTGVA QD)$_2$ specific rabbit antibodies to recombinant protein; Lane G, binding of IgG purified from normal rabbit serum by protein-G column as negative control to recombinant protein.

undetectable (Fig. 3A). Rabbit antisera were diluted up to 1:25600 for P1 and P3, 1:6400 for P2 (Fig. 3B). In western blot analysis, rabbit antisera and three epitope-specific antibodies purified from the antisera by three sepharose columns coupled with the synthetic epitope-peptides P1, P2 and P3 could recognize natural HA from influenza virus particles (strain A/Wuhan/359/95) (Fig. 4, lanes B, C, D and E). In hemagglutination inhibition (HI) tests, these three antisera at the dilutions from 1:20 to 1:80 respectively showed inhibitory activity against influenza virus A/Wuhan/359/95 (Fig. 5). Interestingly, the purified P1 and P2 specific-antibodies could partly inhibit plaque-formation of influenza virus (strain A/Wuhan/359/95) on monolayer MDCK cells, and more than 50% reduction was shown at 10 μg/ml of these antibodies, while the P3 specific antibodies showed a lower inhibitory effect (Fig. 6). Besides these purified antibodies, these antisera could also inhibit plaque-formation of this influenza virus, at dilutions from 1:100 up to 1:1000 (Fig. 7).

Discussion

Antigenic variation of HA is one of the most important characteristics of influenza virus (Wilson & Cox, 1990). Only one amino acid mutation in each antigenic site of HA may generate a new epidemic strain (Wiley et al., 1981). Currently used vaccines must be amended annually to follow the rapid change of epidemic strains. The main difficulty in design of a new influenza vaccine is to develop one that is capable of inducing a broad antibody response protective against infection by heterologous viruses. Many synthetic vaccines have been studied for induction of the neutralizing capacity, but the main trend is to develop a better component or more efficient adjuvant (Levi et al., 1995; Levi & Arnon, 1996). We suggested epitope vaccine as a new strategy against viral infection (Chen et al., 1999; Xiao et al., 2001). An epitope vaccine comprising repeats of linear neutralizing B cell epitope had improved the immunogenicity of this epitope and induced a potent neutralizing antibody response (Lu et al., 2002). We have succeeded in synchronously inducing high levels of antibodies with predefined multi-epitope-specificity by a recombinant multi-epitope-vaccine (Li et al., 2002). In this study, the recombinant immunogen bearing three predefined epitopes of HA was identified by three

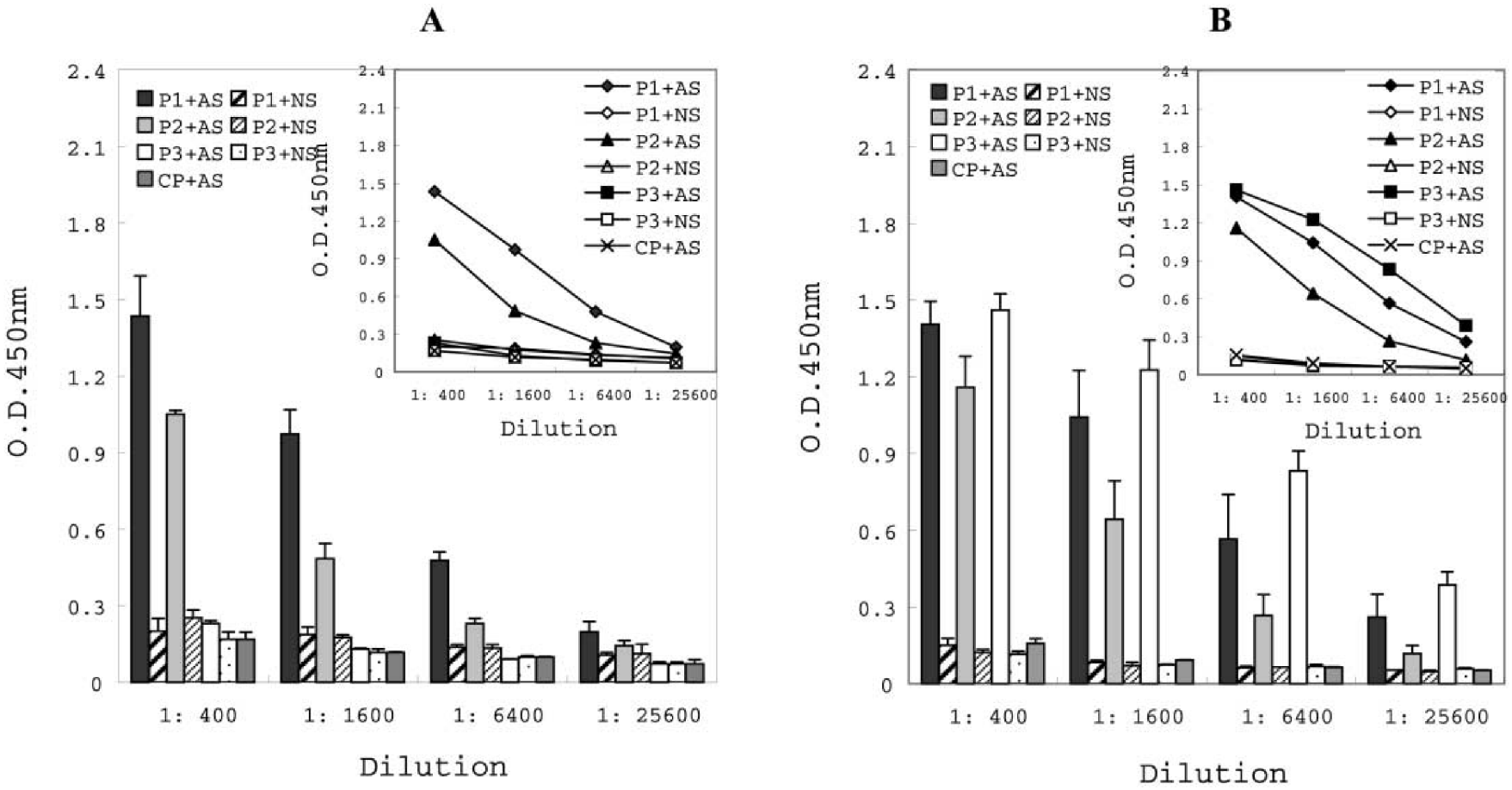

Fig. 3. Detection of the epitope-specific antibodies in mice (A) and rabbits (B) antisera induced by the recombinant immunogen in ELISA-assay. AS: antisera; NS: normal sera (pre-immune sera); P1: C-(KAYSNCYPYDVPDY*G*)$_2$ (HA aa92–105); P2: C-(*G*HHPITDSDQTRLY)$_2$ (HA aa183–195); P3: C-(*G*WTGVAQD)$_2$ (HA aa127–133); CP: control peptide [(KGGG)$_7$-K]. Results from three sera were expressed as means. The figure shows the data from one of three separate experiments.

epitope-specific antibodies in western blot analysis (Fig. 2). The recombinant immunogen elicited a strong humoral immune response with multi-specificities in mice and rabbits, the serum dilutions from 1 : 6400 to 1 : 25600 were confirmed in ELISA assay.

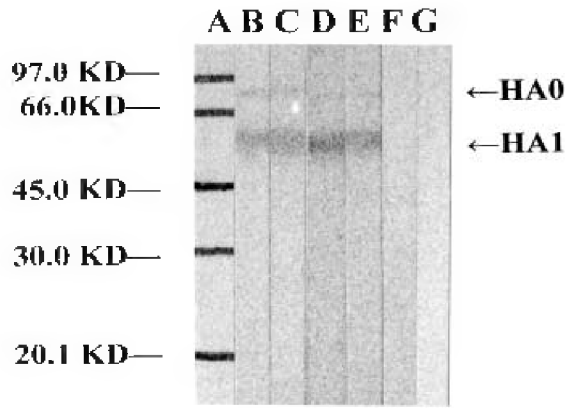

Fig. 4. Binding of antiserum and three epitope-specific antibodies to natural HA in western blot analysis. Three epitope-specific antibodies were purified from rabbit antisera against the recombinant immunogen by three sepharose columns coupled with the synthetic epitope-peptides P1, P2 and P3. Virus particles of strain A/Wuhan/359/95 were subjected to electrophoresis and electroblotted onto NC membranes. Lane A, protein molecular weight maker; Lane B, rabbit antiserum induced by multi-epitope protein bind to HA; Lane C, P1-epitope specific antibodies bind to HA; Lane D, P2-epitope specific antibodies bind to HA; Lane E, P3-epitope specific antibodies bind to HA; lane F, rabbit pre-immune sera did not bind to HA; Lane G, IgG purified from pre-immunized sera by protein-G column did not bind to influenza proteins.

These antisera and the purified epitope-specific antibodies could recognize natural HA in blotting tests. In a functional study, these three antisera at the

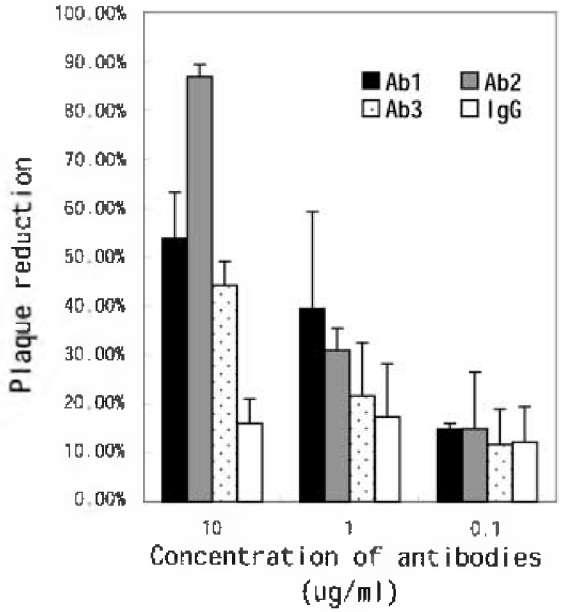

Fig. 6. Inhibition of plaque formation by epitope specific antibodies. Three epitope-specific antibodies (Ab1, Ab2 and Ab3) were purified from rabbit antisera against the recombinant immunogen by three sepharose columns coupled with the synthetic epitope-peptides P1, P2 and P3. IgG as negative control was isolated by protein-G column from rabbit pre-immune sera. The figure shows the data from one of three separate experiments.

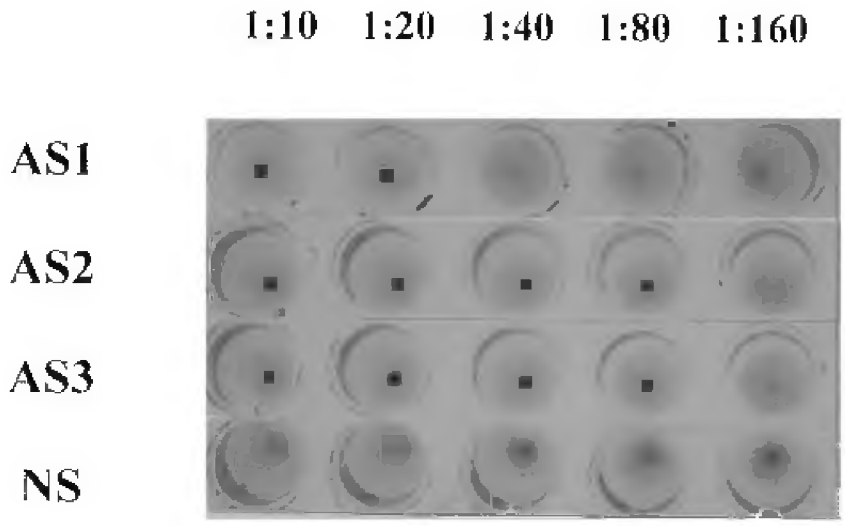

Fig. 5. Hemagglutination inhibition by rabbit antiserum induced by multi-epitope vaccine. All the antisera and the pre-immune sera were treated with trypsin and $NaIO_4$ to reduce non-specific agglutinins and non-specific agglutination inhibitors respectively before use in the HI assay. Viral suspensions were prepared to contain 4 HUs per 25 μl PBS. Sera were serially diluted with PBS. Chicken RBCs in PBS were used to clarify the HI titres of the antisera and the pre-immune sera. Triple wells were repeated at each dilution. The figure shows the data from one of three separate experiments.

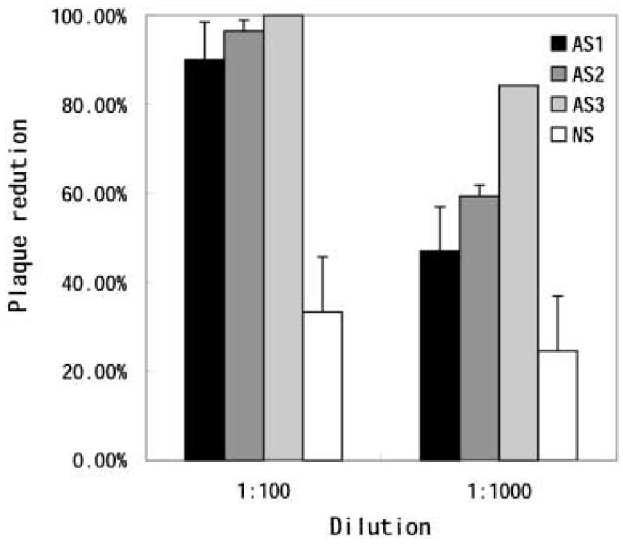

Fig. 7. Inhibition of plaque formation by antiserum induced by multi-epitope vaccine. Antisera of three rabbits and pre-immune serum were treated with trypsin and $NaIO_4$ to reduce non-specific agglutinins and non-specific agglutination inhibitors before use. The figure shows the data from one of three separate experiments.

dilutions from 1:20 to 1:80 showed inhibitory activity in the hemagglutination inhibition test. These antisera and the purified epitope-specific antibodies could partially inhibit plaque-formation of influenza virus. These results suggest that the recombinant multi-epitope vaccine can simultaneously induce multi-antiviral activities against influenza virus, which may provide a new way to develop an effective vaccine against influenza virus.

Experimental data from animal models showed that antibody response induced by whole influenza virion immunization might focus on only one epitope of HA (Lambkin & Dimmock, 1995, 1996). This bias was more obvious after second and third immunization and did not broaden. The human immune system behaved in a similar way. By this token, the recombinant multi-epitope vaccine showed predominance by synchronously inducing a high level of antibodies with predefined multi-epitope-specificity. Wang et al. (1986) found a relatively restricted response in human sera taken between 1969 and 1971 but not in sera taken in 1978. This indicated that mutations in one antigenic site might lead to virus escape in a subpopulation. A vaccine that can induce neutralizing antibodies against two or more different antigenic sites may dramatically decrease the possibility of escape. Sequence analysis within the H3N2 subtype had revealed the high conservation rate of all the sites of the HA1 epitope aa92–104 with only one restricted variable residue (Lu et al., 2002). Five more variable sites with restricted mutations were found in the other two HA1 epitopes aa127–133 and aa183–195 while the others are highly conserved (data not shown). As the candidate multi-epitope vaccine had shown an advantage in synchronously inducing antibodies with multi-epitope-specificities, broad protection against infection of heterogeneous viruses might be expected by the multi-epitope-specific immune response induced by a candidate vaccine with more epitopes including these three epitopes.

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