Production of anti-dengue NS1 monoclonal antibodies by DNA immunization

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Abstract

Monoclonal antibodies against dengue NS1 protein were generated following immunization of mice with plasmid DNA encoding the transmembrane form of NS1 from dengue serotype 2 virus. A mammalian expression vector, pDisplay, was engineered to direct cell surface expression of dengue NS1 and tested for transient expression in COS cells. Two mice were immunized intramuscularly with six doses of 100 μg of plasmid at 2-week intervals; one mouse received a booster of live virus prior to the last plasmid injection. Both mice showed antibody responses against dengue antigens in dot enzyme immunoassay. Following fusion, hybridomas were screened with dot enzyme immunoassay against all four dengue serotypes. Specificity to the NS1 protein was confirmed by western blot analysis. Among five anti-dengue NS1 monoclonal antibodies generated, two clones were serotype 2 specific, two clones reacted with all four serotypes and the last also reacted with Japanese encephalitis virus. Reactivity against native or denatured forms of NS1 revealed three clones with reactivity to linear epitopes and two clones recognizing conformational epitopes. Such diverse specificity of anti-dengue NS1 monoclonal antibodies indicates that DNA immunization, especially with the combination of virus boosting, is an efficient way of producing monoclonal antibodies against viral protein. This has opened up a possibility of producing monoclonal antibodies to rare viral proteins that are difficult to isolate or purify.

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1. Introduction

Dengue virus contains a positive polarity, single-stranded RNA genome of approximately 11 kb in length. A single polyprotein was co-translationally processed by viral and cellular proteases into three structural proteins; capsid, prM, and envelope (E), and seven non-structural proteins; NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5 (Chang, 1997). Existing monoclonal antibodies against the dengue proteins available worldwide are mainly those directed against E, NS1 and prM proteins. These clones were obtained by immunization with either dengue virus or dengue recombinant proteins (Falconar and Young, 1991; Gentry et al., 1982; Henchal et al., 1987; Kaufman et al., 1989, 1987; Pupo_Antunez et al., 2001; Roehrig et al., 1998). Antibodies against other dengue proteins are rare, partly due to the difficulties in preparing adequate quantity of antigens for immunization.

During the 1990s, in vivo expression of genes encoding exogenous proteins in mice and its efficiency in inducing specific antibody responses following naked DNA injection were reported (Tang et al., 1992; Wolff et al., 1990). As an alternative approach to the genera-
tion of immune response, DNA immunization has been shown to elicit both cellular and humoral immunity against several infectious pathogens as well as allergens and cancers (Hasan et al., 1999). Its advantages and proposed mechanisms of enhancing immunity have been extensively reviewed (Hasan et al., 1999; Hassett and Whitton, 1996; Whitton et al., 1999). When compared with conventional vaccines, DNA vaccine is safer (from lacking a replicative agent), relatively cheap to produce, heat stable, amenable to genetic manipulation and capable of inducing long-term cellular immunity (Gurunathan et al., 2000). In flaviviruses, DNA vaccines against Dengue 2 virus (Porter et al., 1998), Murray Valley encephalitis virus (Colombage et al., 1998), St Louis encephalitis virus (Phillpotts et al., 1996) and Japanese encephalitis virus (Konishi et al., 1998) were shown to be efficient in inducing antibody responses and induced full or partial protection in mice against virus challenge.

Besides vaccine application, DNA immunization strategy can be another efficient method for the purpose of production of polyclonal and monoclonal antibodies (Barry et al., 1994; Kasinrerk and Tokrasinwit, 1999; Kasinrerk et al., 1997, 1996; Moonsom et al., 2001; Ulivieri et al., 1996). In the present study, we attempted to use DNA immunization for the generation of monoclonal antibodies against a dengue non-structural protein and we demonstrated that plasmid DNA containing gene encoding dengue viral NS1 protein could be efficiently used to produce anti-NS1 monoclonal antibodies. The antibodies are reactive to different epitopes similar to those produced by conventional immunization using viral or purified antigens.

2. Materials and methods

2.1. Cell lines and hybridomas

COS seven cells were maintained in Minimum Essential Medium (MEM; Gibco-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL) and 40 μg/ml gentamicin and 2.5 μg/ml amphotericin B in a humidified atmosphere of 5% CO₂ at 37 °C. PS clone D cells were maintained in L-15 media (Gibco-BRL) containing 10% FBS, 10% tryptose phosphate broth (Gibco-BRL) and 1.2% streptomycin and penicillin antibiotics (Sigma, St.Louis, MO, USA) at 37 °C. C6/36 cells were maintained in the same media as PS clone D but were incubated at 28 °C. During dengue virus infection of C6/36 and PS clone D cells, FBS content in L-15 media was reduced to 1%.

Hybridoma clones 1B2 (specific for dengue NS1), 4G2 and 3H5 (specific for dengue E) and 2H2 (specific for dengue prM) were grown in RPMI 1640 media (Gibco-BRL) containing 10% FBS and 1.2% streptomycin and penicillin antibiotics (Sigma) at 37 °C in 5% CO₂.

2.2. Viruses

Dengue viruses serotype 1 (strain Hawaii), serotype 2 (strain 16681), serotype 3 (strain H87) and serotype 4 (strain H241) were propagated in C6/36 mosquito cell line. Culture supernatant containing dengue virions as well as secreted NS1 protein was harvested 4–5 days after infection. Japanese encephalitis virus (strain Nakayama) was obtained from Dr Ananda Nisalak, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand and propagated in C6/36 cells.

2.3. Construction of recombinant plasmid

A 1056-bp NS1 gene segment was obtained by BamHI/SalI double digestion from a pMal/NS1 plasmid, which has been constructed from reverse transcriptase–polymerase chain reaction product of dengue type two virus NGC strain (S. Pattanakitsakul, unpublished data), ligated into the Bg/II/SalI site of a mammalian expression vector pDisplay (Invitrogen, Carlsbad, CA) and transformed into DH5αF′. Selection of E. coli colonies containing the recombinant plasmid, pDisplay/sNS1tm, was performed by colony polymerase chain reaction and restriction enzyme digestion. The pDisplay/sNS1tm plasmid was extracted and purified by Qiagen plasmid purification kit (Qiagen, Hilden, Germany).

2.4. DEAE–dextran mediated transfection

COS cells were plated in a 60-mm tissue culture dish (Nunc, Roskilde, Denmark) at 7.5 × 10⁵ cells/dish and incubated in 5% CO₂ at 37 °C for 24 h culture media was removed and cells were transfected with 2 ml of MEM containing 250 μg/ml DEAE-dextran (Sigma), 400 μM chloroquine diphosphate (Sigma) and 5 μg plasmid DNA for 3 h at 37 °C in 5% CO₂. Supernatant was removed and cells were treated with 10% dimethyl sulfoxide in phosphate buffered saline (PBS) for 2 min at room temperature. Cells were then washed once with MEM, replaced with MEM containing 10% FBS and incubated in a 5% CO₂ incubator at 37 °C. The culture media was changed on the next day. Transfected cells were harvested at 62 h after transfection and assayed for the expression of dengue NS1 by indirect immunofluorescence.

2.5. Indirect immunofluorescence assay

Transfected COS cells were detached from culture dish using PBS containing 0.5 mM EDTA and washed twice with PBS containing 1% bovine serum albumin
and 0.02% sodium azide. Cells were then incubated with known anti-dengue NS1 monoclonal antibody for 30 min on ice. After washing, cells were incubated with rabbit anti-mouse immunoglobulin-FITC conjugate (Dako, Glostrup, Denmark) for 30 min. Membrane fluorescence was detected using a fluorescence microscopy (Olympus, Tokyo, Japan).

2.6. Western blot analysis

Transfected COS cells were harvested and resuspended in PBS. Cells were lysed in 4 × reducing sample buffer and heated at 95 °C for 5 min. Cell lysate was then separated with 10% SDS-PAGE and blotted onto nitrocellulose membrane. The membrane was blocked with 5% skimmed milk in PBS and reacted successively with 1B2 monoclonal antibody and horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dako). The antigen–antibody complex was visualized with H2O2 and diaminobenzidine.

Reactivity of hybridoma culture supernatants against different forms of NS1 was determined by western blot analysis. Dengue serotype 2-infected C6/36 cells were lysed with 1% Triton X-100 in PBS. Prior to electrophoretic separation, cell lysate was either not treated or subjected to 2-mercaptoethanol treatment at room temperature or at 95 °C for 5 min. Subsequent steps in electrophoresis, blotting and immunological reactions were as described above.

2.7. DNA immunization and hybridoma generation

Two 6-week-old, female Balb/c mice were injected with 50 μl of 1 mg/ml pDisplay/sNS1tm into each hind leg for five times with 2-week interval. One mouse was given the last inoculation with the same dose of plasmid DNA whereas the other received 100 μl of live dengue two virus (5 × 10^5 pfu/ml) and then followed by plasmid DNA as the last immunization dose 2 weeks later. Blood samples were collected just prior to the first immunization and at 2 weeks after each injection by tail bleeding. Mouse sera were separated and stored at −20 °C. Seven days after the last immunization, mice were sacrificed and spleen cells were fused with P3-X63Ag8.653 myeloma cells using 50% polyethylene glycol (Sigma). After HAT medium selection, culture supernatants were analyzed for antibody reactivity against viral protein using cell-ELISA and dot enzyme immunoassay. Hybridomas were cloned by limiting dilution and their isotypes determined by using mouse monoclonal ELISA isotyping kit (Sigma).

2.8. Cell-ELISA

PS clone D cells (5 × 10^6 cells in 100 μl) were plated into a 96-well tissue culture plate, infected with dengue serotype 2 virus at the multiplicity of infection of 1.0. After 24 h of incubation at 37 °C, cells were washed with PBS, fixed with 3.7% formaldehyde for 10 min, and permeabilized with 1% Triton X-100. Anti-dengue antibodies in mouse sera or hybridoma supernatants were detected by adding 50 μl of diluted mouse sera or neat supernatant into permeabilized PS monolayer in both infected wells and mock-infected wells and incubated for 1 h at room temperature. Cells were washed three times with PBS containing 0.05% Tween-20 and reacted with rabbit anti-mouse immunoglobulins-horse radish peroxidase conjugate (Dako). Following additional washing steps, H2O2 and OPD were added and the reaction was stopped with 4 N H2SO4. Optical density was spectrophotometrically determined at 492 nm. Reactivity of all tested samples was subtracted with mean optical density value of mock-infected wells and the corrected value of 0.100 or greater was considered positive. Known monoclonal antibodies against dengue E protein (4G2 and 3H5), NS1 protein (1B2) as well as prM protein (2H2) was included in every cell-ELISA plate.

2.9. Dot enzyme immunoassay

For hybridoma screening assay, a mixture of culture supernatants of C6/36 cells infected with each of the four dengue serotypes and mock-infected supernatant were dotted onto nitrocellulose membrane, blocked with 5% skimmed milk in PBS and reacted with mouse sera (1:50 dilution) or hybridoma supernatants (1:5 dilution) and incubated for 1 h at 37 °C. After washing with PBS, bound antibody was detected with rabbit anti-mouse immunoglobulins-horse radish peroxidase conjugate (Dako) and H2O2–diaminobenzidine–0.04% NiCl2 mixture. Positive reaction appeared as dark brown dot on the membrane was graded from 0, 1+, 2+, 3+ and 4+. For determining the cross reactivity of the anti-NS1 monoclonal antibodies, the antibodies were tested against a nitrocellulose membrane that contained six dots of dengue virus serotype 1–4. Japanese encephalitis virus and mock infected C6/36 cell culture supernatants.

3. Results

3.1. Expression and immunogenicity of dengue NS1 fusion plasmid

A 1.056-kb NS1 fragment, containing nt 2422–3477 from dengue serotype 2 genome, was ligated into pDisplay to create a fusion gene containing a leader peptide sequence derived from the murine Igk light chain, an extracellular portion of NS1 and a transmembrane anchor sequence from platelet-derived growth factor receptor. Expression of this fusion gene in
mammalian cell was tested by transfecting recombinant plasmid into COS cells and identifying cell surface NS1 with an anti-dengue NS1 monoclonal antibody, 1B2. Within 62 h after transfection, 1B2 epitope was clearly detected by an indirect immunofluorescence method on the cell surface in about 5% of cells (Fig. 1). Under the same conditions, mock-infected COS cells did not react with 1B2. When transfected COS cell lysate was treated with 12% sodium dodecyl sulfate in the presence of reducing agent and subjected to western blot analysis, a protein band of approximately 57 kDa was identified in plasmid-transfected COS lysate, but not mock-infected lysate (Fig. 2). As expected, NS1 fusion protein expressed in transfected COS cells was slightly larger than 48-kDa native NS1 found in dengue-infected mosquito cells (Fig. 3A, lane 4). These results indicate that the NS1 fusion protein can be expressed on mammalian cell surface.

When two Balb/c mice were immunized with NS1 fusion plasmid at 2-week intervals and anti-dengue antibody response was monitored by dot enzyme immunoassay. Significant level of anti-dengue antibodies was detected equally in both mice (dot 1+ in a scale of 4, with no background in the control dot) after the third plasmid injection and continued up to the end of immunization schedules. Comparison of the antibody responses between both mice just before fusion revealed that the mouse that had been additionally boosted by live virus injection had higher antibody level (dot 2+) than the one boosted by plasmid only (dot 1+). Thus, immunization of plasmid DNA encoding dengue NS1 fusion protein can induce antibody response in mice.

3.2. Generation and characterization of anti-NS1 monoclonal antibodies

Following fusion of spleen cells from plasmid-immunized mice and selection using standard hybridoma technique, 17 out of 218 hybridoma clones (7.8%) from DNA immunized mouse and 11 out of 71 hybridoma clones (15.5%) from virus-boosted mouse were reactive to dengue antigens either by dot enzyme immunoassay or cell-ELISA. After cloning by limiting dilution, five hybridoma clones, designated NS1-1F, NS1-2F, NS1-3F, NS1-4F and NS1-2S, were selected based on their reactivity against dengue NS1 protein by western blot analysis (Table 1). The first four clones were derived from mouse immunized with DNA plus virus boost whereas NS1-2S was from DNA-immunized mouse.

![Fig. 1. Photomicrograph of pDisplay/sNS1tm-transfected COS cells. COS cells were transfected with pDisplay/sNS1tm for 62 h and stained with anti-NS1 clone 1B2 and rabbit anti-mouse immunoglobulins-FITC conjugate. Cells were observed under light microscope (A) or fluorescence microscope (B).](image)

![Fig. 2. Western blot analysis of pDisplay/sNS1tm-transfected COS cell lysate. Lane 1, protein markers with indicated molecular weight in kDa on the left side; lane 2, COS cells were transfected with pDisplay/sNS1tm for 62 h, and subjected to western blot analysis employing anti-NS1 clone 1B2. Cells were treated with reducing agent and heated prior to electrophoresis.](image)
mouse. All clones were reactive to dengue antigen by dot enzyme immunoassay but only three of them reacted in cell-ELISA (NS1-1F, NS1-3F and NS1-4F). Three clones were of IgM class (Table 1).

Within dengue-infected cells, NS1 associates non-covalently into homodimeric form approximately 20–40 min after its synthesis (Winkler et al., 1988). With heat treatment, dimeric NS1 readily dissociates into monomeric form (Winkler et al., 1988). To investigate the reactivity of monoclonal antibodies against the two forms of dengue NS1, dengue-infected cell lysate was employed in western blot analysis either without any additional treatment, with heat treatment, with 2-mercaptoethanol treatment, or with heat and 2-mercaptoethanol treatment. Clones NS1-1F, NS1-3F and NS1-4F reacted with dimeric NS1 of approximately 66 kDa under non-reducing condition (Fig. 3A, lane 1) and 90 kDa under reducing condition (Fig. 3A, lane 3). They also reacted with the monomeric form of 45 and 48 kDa under non-reducing and reducing conditions, respectively (Fig. 3A, lanes 2 and 4). This reactivity pattern was similar to that of 1B2, indicating that NS1-1F, NS1-3F and NS1-4F recognize a linear epitope on NS1. In contrast, clones NS1-2F and NS1-2S did not bind NS1 after heat and 2-mercaptoethanol treatment (Fig. 3B, lane 4); both of them are, therefore, specific for conformational epitopes that are absent in the reduced monomeric form of NS1.

To determine cross-reactivity of the five anti-NS1 clones to other dengue serotypes and Japanese encephalitis virus, dot enzyme immunoassay utilizing supernatants obtained from C6/36 cells infected with each serotype of dengue and Japanese encephalitis viruses was performed. As shown in Table 1, these anti-NS1 antibodies were classified into three groups depending on their reactivity with tested viruses. The first group, NS1-1F, appeared to be flavivirus cross-reactive as it bound all four serotypes of dengue as well as Japanese encephalitis virus. The second group, NS1-3F and NS1-4F, reacted with the four dengue serotypes but not Japanese encephalitis virus whereas the last group, NS1-2F and NS1-2S, appeared to be dengue serotype 2 specific.

4. Discussion

In several laboratory animals, DNA immunization has been shown to be an efficient mean for induction of both humoral and cell-mediated immune responses. In this report, we employed a recombinant plasmid encoding dengue NS1 fusion protein to aid in the generation of monoclonal antibodies to NS1 protein. A mammalian expression vector, pDisplay, was used as this vector contains a high-level expression promoter derived from cytomegalovirus; it has been used previously in DNA vaccine development against many infectious pathogens (Hasan et al., 1999). An advantage to the use of

<table>
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<th>Designation</th>
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<th>Isotype</th>
<th>Epitope type</th>
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<tr>
<td>NS1-1F</td>
<td>DNA + virus</td>
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<td>Linear</td>
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<tr>
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<tr>
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<td>Dengue serocomplex</td>
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<tr>
<td>NS1-4F</td>
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<td>IgG1</td>
<td>Linear</td>
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<tr>
<td>NS1-2S</td>
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pDisplay is the expression of target protein on cell surface. Several reports suggested that antigens presented on the cell surface are often more immunogenic than intracellular or secreted forms (Haddad et al., 1995; Men et al., 1991; Srinivasan et al., 1995). In a direct comparison study, DNA immunization with pDisplay containing hepatitis C virus E2 protein, which the expressed E2 targeted to the cell surface, induced much stronger humoral immune response in mice and macaques than pcDNA3.1 vector carrying the same gene but expressed E2 protein intracellularly (Forns et al., 1999). Interestingly, while immunization with plasmid DNA alone is adequate for the generation of anti-dengue NS1 monoclonal antibody, an additional boost with live virus injection appears to increase the number of clones obtained. Although the number of NS1-specific clones generated with the two immunization schedules are obviously small for comparison, our finding agrees well with previous reports showing enhancement of specific immune responses after antigen boost following DNA immunization (Schmolke et al., 1998; Tearina_Chu et al., 2001).

The monoclonal antibodies against dengue NS1 generated by DNA immunization in this study are quite diverse with regards to isotype, the nature of epitope recognized and cross-reactivity to NS1 from other viruses. These results suggest that the NS1 fusion protein expressed in murine cells following injection of plasmid DNA closely resembles those of native NS1 protein despite the facts that mouse is not a natural host for dengue virus and NS1 localized on the surface of dengue-infected human cells is in the glycosyl-phosphatidylinositol-linked form (Jacobs et al., 2000). Possible differences in the post-translational modifications of NS1 within murine cells as compared with infected human cells appear not to affect the immunogenicity of fusion NS1 protein generated in mice. However, we are also aware of the fact that the diversity of the antibodies obtained from the mouse that had been boosted by natural virus might also be generated by the exposure to the virus itself.

In conclusion, we have demonstrated that it is possible to produce monoclonal antibodies against the dengue NS1 protein by using DNA immunization strategy. The monoclonal antibodies are shown to be reactive to different natural epitopes of the NS1. This has opened a possibility of producing additional clones against other proteins that, in natural conditions, are expressed in small amount and/or difficult to purify or prepare. Using this similar technique, we have successfully produced antibodies to prM protein of dengue virus (manuscript in preparation). The monoclonal antibodies obtained from this report will be useful for further development of dengue NS1 diagnosis as well as functional study of dengue NS1 protein.

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