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Immunization with DNA Vectors Consisting of Selected Dengue 2 Virus Genes Stimulated Antibody Responses in Mice

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Abstract: Dengue virus Type 2 C, prM, truncated E (NB-E and B-E), NS1, NS2A, NS2B/3 and NS3 genes were cloned and expressed using the pEGFP-N1 eukaryotic expression vector. Protein expression was visualized in transfected Vero cell cultures. Immunogenicity of the recombinant DNA plasmids was determined by inoculating 8 weeks old BALB/cJ mice with two doses of plasmid cocktail followed by a booster inoculation at two weeks apart. Mice were also injected with empty vector plasmids or vaccinated with virus inoculum as controls. Initial inoculation with the plasmid cocktail resulted in IgG response comparable to inoculation with virus inoculum alone which peaked after the booster dose. Rapid rise in IgG response was also observed when the mice were subsequently challenged with partially purified dengue virus Type 2, ten weeks after the first inoculation. These results suggest for the first time, that dengue virus DNA genes expressing antigens as fusion proteins with EGFP could stimulate immune responses *in vivo*.

Key words: IgG response, dengue virus, Recombinant DNA, plasmid, pEGFP-N1, cytotoxic T-lymphocytes

Introduction

Dengue fever and its more serious forms, dengue hemorrhagic fever and dengue shock syndrome, are still a major concern in Malaysia and most Southeast Asian countries (Henchal and Putnak, 1990). The disease is caused by any one of the four dengue virus serotypes. Efforts to limit the infection were centered on development of vaccine and control of mosquito vectors mainly the *Aedes aegypti* (Fenming *et al*., 1996). However, only limited success is achieved in both areas. Successful development of effective vaccine has been hampered primarily due to the lack of understanding on the mechanisms of dengue virus pathogenesis. These include the role of antibody dependent enhancement (ADE) *in vivo* and the contribution of cytokines in the manifestation of severe infections. Even though live attenuated vaccines are inexpensive and their protective effects are persistent, there are risks for reversion in the virulence and introduction of high virulence to immuno-compromised patients. Hence, with the advent of molecular biology, especially in DNA vaccine technology beginning in the early 1990's (Wolf *et al*., 1990; Tang *et al*., 1992), it is conceivable that dengue virus specific DNA sequences encoding specific antigenic peptides be engineered and used as a powerful, safer and more effective vaccines.

Previous reports on DNA vaccination in mice and monkeys were shown to be successful (Konishi *et al*., 2000; Raviprakash *et al*., 2000, Kochel *et al*., 2000). These studies, however, used only

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specific genes of the virus genome particularly the prM and E structural proteins which are known to be immunogenic. Lately, the nonstructural but highly immunogenic NS1 protein also has been an alternative for vaccine development (Srivastava *et al*., 1995). The protein is an important vaccine candidate since antibodies directed to NS1 will not affect ADE (Winkler *et al*., 1988). Other major nonstructural proteins, NS3 and NS5 also elicited humoral response in dengue patients (Valdes *et al*., 2000), however, the levels were trivial compared to NS1. In addition, human CD8+ cytotoxic T lymphocytes have been shown to have recognition of dengue virus nonstructural proteins NS3 and NS1.2a (Mathew *et al*., 1996). Based on these and other reports we propose to investigate the prospect of using a cocktail mixture of selected dengue virus genes as a vaccine to minimize potential incidence of ADE. The long-term objective of these studies was to develop an effective tetravalent dengue DNA vaccine. Towards this goal we focused our efforts in the construction and evaluation of a monovalent (dengue-2) vaccine. In the present study, mice were inoculated with a cocktail mixture of dengue Type 2 DNA vaccine containing dengue virus genes engineered into the pEGFP-N1 eukaryotic expression vectors and the immune responses were measured. The DNA vaccine expresses the dengue 2 viral antigens as fusion proteins with EGFP (Cormack *et al*., 1996). Data are presented here which suggest successful stimulation of antibody responses in mice inoculated with the DNA vaccine.

Materials and Methods

Cloning of Dengue Virus Genes

Dengue 2 virus C, prM, NB-E, B-E, NS1, NS2A, NS2B/3 and NS3 genes were amplified by the RT-PCR method using specific primers. The amplified fragments were then cloned individually into the pGEM-T cloning vector (Promega, USA). Following transformation, positive transformants were amplified and plasmids were purified and digested using *Bam*H I and *Not* I restriction enzymes (Shafee and AbuBakar, 1999). The resulting fragments were then ligated into the pEGFP-N1 eukaryotic expression vector (Clontech laboratories, Inc., USA) in frame with the EGFP gene. Plasmid constructs were verified by restriction enzyme digestion and DNA sequencing analysis of the gene insert.

Plasmid Purification and Transfection

Plasmids were purified using EndoFree™ Plasmid Maxi Kit (QIAGEN Inc., USA) following the manufacturers protocol and resuspended in endotoxin-free PBS (Sigma-Aldrich Ltd., UK) at a final concentration of 1 μg μL^{-1}. The plasmids suspensions were then transfected into Vero cells using the Tfx-20 transfection reagent (Promega, USA). Initially, Vero cells were cultured overnight in 24 wells tissue culture plates containing sterile 13 mm^2 glass coverslips (Menzel-Glaser, Germany) at a density of 5×10^4 cells per well. Each of the plasmid suspension was mixed by vortexing with serum free medium at a final concentration of 5 μg mL^{-1}, followed by addition of 10.8 μL Tfx-20 reagent. The mixture was immediately vortexed again and incubated at 25°C for 10 min. In the mean time, growth media from the cells were removed and 200 μL of the medium/DNA/Tfx-20 reagent mixture was added. The plates were incubated at 37°C for 1 h followed by addition of 1 mL complete medium and further incubation at 37°C for 48 h. *In vitro* expression of the recombinant plasmids was assayed by detecting the fluorescent EGFP protein fused to the carboxy terminal ends of the recombinant proteins at excitation and emission maxima at 488 and 507 nm, respectively.

Plasmid Inoculation into Mice

Upon confirmation of protein expression, two groups of eight weeks old BALb/cJ mice were inoculated intramuscularly with 100 μg of the recombinant plasmid DNA cocktail at two weeks apart. The mice were bled two weeks following each dose and the resulting sera were kept at -20°C until further use. Concurrently, 100 μL of dengue 2 virus inoculum (5×10^4 pfu mL^{-1}), 100 μg of the pEGFP-N1 circular plasmids and 100 μL of endotoxin-free PBS were also inoculated into mice and sera were harvested as controls. Six weeks after the last dose, the mice were challenged with 100 μL partially purified virus (5×10^5 pfu mL^{-1}). Sera were collected at selected intervals for one month post-challenge and the antibody responses were analysed by ELISA.

Assay for Immune Responses

IgG-specific ELISA was performed on all the collected sera using dengue 2 virus-infected C6/36 cell lysate as antigen. The lysate was used to coat the 96-wells microtiter plates at a concentration of 2 μg $well^{-1}$ in 50 mM, pH 9.9, Na_2CO_3 buffer for 3 h at 25°C. Following the incubation, the plates were washed with PBS containing 0.05% Tween-20 (PBS-T) and blocked with 3% BSA in PBS for 1 h at 25°C. The plates were finally washed with PBS-T and 150 μL of 1:40 dilution (in 1% BSA/PBS-T) of the collected sera were added. The plates were further incubated at 25°C for 3 h. Alkaline phosphatase-conjugated goat anti-mouse IgG (Promega Inc., USA) was used as a secondary antibody and developed using phosphatase substrate for ELISA (KPL Inc., USA). The reaction was terminated with 5% EDTA solution and the absorbance was noted at a wavelength of 410 nm.

Results and Discussion

Successful expression of the fusion proteins in Vero cells transfected with each of the dengue 2 virus C, prM, NB-E, B-E, NS1, NS2A, NS2B/3 and NS3 -EGFP plasmid clones was confirmed by detection of the fluorescent EGFP under a ultraviolet light microscope after 48 h post-transfection (Fig. 1a). Even though equal concentration of each clones were used in the transfection, the efficiency varies (Fig. 1b) giving an average value of 30(±6)% of positive transformants. The equal concentrations of the recombinant plasmids in the cocktail mixture were maintained to allow equal probability for protein expression *in vivo*. Nevertheless, expression of the recombinant proteins in Vero cells suggested that the plasmid clones were intact and antigens were expressible in the inoculated mice. Utilization of EGFP as a reporter protein in DNA vaccination has been reported (Quinn *et al*., 2002; Perkins *et al*., 2005). Our work, however, is the first to report on the use of antigen-EGFP fusion proteins in DNA vaccines against dengue virus infections. The EGFP component of the proteins appeared to present no interfering responses to the immunogenecity of the viral protein antigens. This is evident from the levels of protective immunity in the present study which was parallel to those in previous reports of dengue DNA vaccination (Konishi *et al*., 2000; Raviprakash *et al*., 2000).

Immunization with the DNA vaccine cocktail resulted in increased IgG response comparable to immunization with the virus inoculum alone (Fig. 2). In each case, the IgG response began to accumulate during the first 2 weeks of injection and peaked (30-fold increase in geometric mean titer) after the third booster dose. A low IgG response was also observed in the pEGFP-N1 plasmid control. This was perhaps due to the nonspecific binding of mouse antibodies to the infected C6/36 cell lysate proteins used as crude dengue virus Type 2 antigen. Previous report on the use of EGFP as reporter for gene transfer and expression also showed slight immune response against EGFP

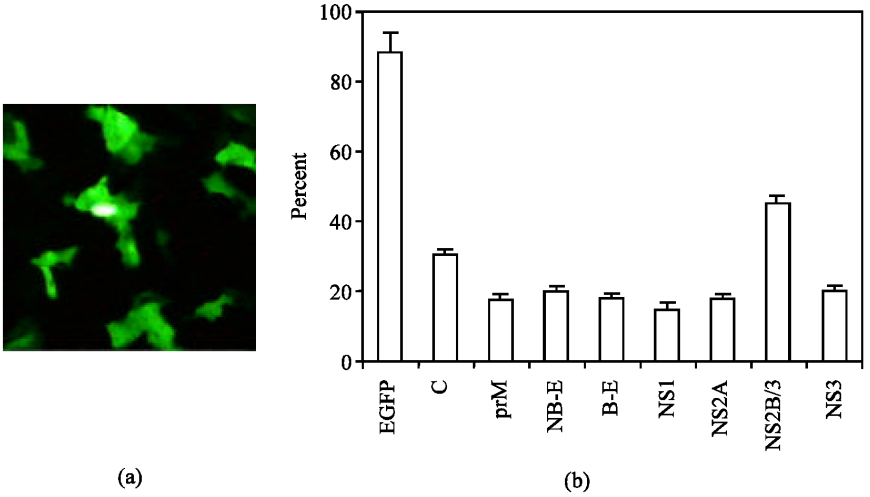

Fig. 1: Expression of EGFP fusion proteins in Vero cells. (a) Successful expression of the fusion proteins was confirmed by detection of the fluorescent EGFP under an ultraviolet light microscope after 48 h post-transfection. Cells were observed under a Zeiss Axiovert25 UV microscope and images were captured using Nikon F3 camera. (b) Transfection efficiency of EGFP fusion proteins in Vero cells. The efficiency was calculated based on 10 different transfection experiments. The values for positive cells for EGFP marker protein are presented as average of ten microscopic fields

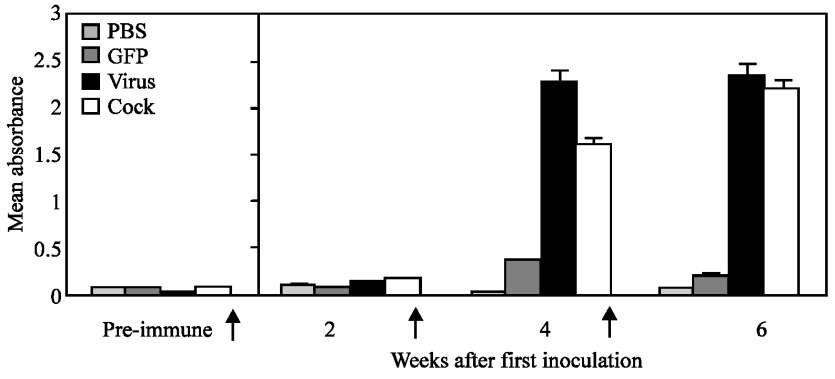

Fig. 2: Detection of IgG specific against dengue 2 virus using ELISA. Arrows indicate time points for DNA inoculation

(Stripecke *et al.*, 1999). No antibody response was observed in the PBS alone suggesting that the diluent used for the plasmid clones were free from any possible contaminants.

To examine if the DNA vaccination induces memory B cells, the vaccinated mice were subsequently challenged with partially purified virus. The response sera were collected at selected

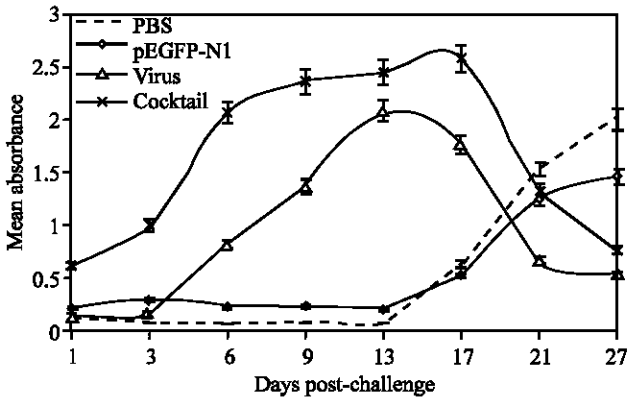

Fig. 3: Detection of IgG specific against dengue 2 virus using ELISA. Six weeks after the last dose of vaccination, mice were challenged with 100 μL of partially purified virus. Sera were collected at selected intervals for one month post-challenge and the antibody responses were analyzed by ELISA

intervals post-challenge. ELISA results on these sera demonstrated that the IgG response for virus-inoculated mice began to be detected after only 3 days post challenge (Fig. 3). This observation is in agreement with previous report on vaccination with a plasmid encoding the Japanese encephalitis virus premembrane and envelope genes (Konishi *et al*., 1999). The study showed that neutralization titers were elevated on day 4 post-challenge. Konishi *et al*. (2000) also reported that neutralizing antibody titers increased beginning on day 4 after challenge with dengue Type 2 virus. This phenomenon was observed in immunization of mice with DNA vaccine expressing dengue Type 2 virus premembrane and envelope genes. Even though the IgG production in the present investigation was accelerated, the level was found to be slightly lower than anticipated for a secondary antigen exposure. The reason was probably due to the C6/36 lysate used as antigen in ELISA which introduced other interfering proteins during binding. Ideally, higher purity antigens such as purified virus should be used.

IgG response in mice immunized with the DNA vaccine was detected earlier and at higher levels compared to the mice immunized with dengue virus inoculum (Fig. 3). This observation is interesting since the immunization was performed without the use of any adjuvants. Commonly used adjuvants include chemical adjuvants such as aluminum hydroxide or aluminum phosphate and immunostimulatory DNA sequences such as CpG motif (Jeong-Im *et al*., 1999; Dory *et al*., 2005). The PBS and pEGFP-N1-vaccinated mice on the other hand demonstrated similar levels of IgG response to that shown earlier demonstrating primary antibody response. Nonetheless, these results suggest that immunization of mice using the plasmid cocktail vaccine confer lasting immunity and show anamnestic antibody response to virus challenge. Hence, this plasmid cocktail could be useful for development of candidate DNA vaccine for application in human.

The IgG response observed here is for the total plasmid cocktail carrying only dengue 2 virus genes. In order to develop a safe and effective tetravalent cocktail DNA vaccine, it is crucial to know which recombinant protein triggers the highest immune response. We are currently pursuing this investigation by inoculating mice with each individual plasmid and monitoring the immune response.

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