

Active immunization by a dengue virus-induced cytokine

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SUMMARY

Dengue type 2 virus (DV)-induced cytotoxic factor (CF) is capable of reproducing various pathological lesions in mice that are seen in human dengue. The present study was undertaken to investigate the protective effect of active immunization of mice with CF. Mice were immunized with 5 μg of CF and prevention of CF-induced increase in capillary permeability and damage to the blood–brain barrier were studied at weekly intervals, up to 48 weeks, by challenging with 3 μg of CF. Maximum protection against increase in capillary permeability and damage to the blood–brain barrier was observed in week 4 after immunization. A breakthrough in the protection occurred with higher doses of CF in a dose-dependent manner. Challenge with a lethal intracerebral (i.c.) dose of DV showed significantly prolonged mean survival time and delayed onset of symptoms of sickness in the immunized mice compared with the normal mice, but the titre of the virus in the brain was similar in the two groups. On i.p. challenge with the virus the protection against damage to the blood–brain barrier was $86 \pm 7\%$ at week 4 and $17 \pm 4\%$ at week 26 after immunization. Sera obtained from the immunized mice showed the presence of CF-specific antibodies by ELISA, Western blot, and by neutralization of the cytotoxic activity of CF *in vitro*. The present study describes successful prevention of a cytokine-induced pathology by specific active immunization.

Keywords cytokine active immunization dengue virus disease protection

INTRODUCTION

Cytokines act in a network of communication signals between different types of cells. Under normal conditions the blood concentration of cytokines is usually low, and they are quickly eliminated or rendered inactive. Under pathological conditions higher levels of different cytokines have been noted; however, their role in the pathogenesis of disease is convincing for some of them only. For example, the role of tumour necrosis factor (TNF) in cerebral malaria [1–3], meningococcal septicaemia [4] and lethal infection of *Escherichia coli* in baboons [5] is known. Other cytokines which may produce pathological effects are IL-1 [6], IL-6 [7], IL-8 [8] and others [9].

Dengue haemorrhagic fever/shock syndrome (DHF/DSS) is characterized by increased vascular permeability, thrombocytopenia, alterations in blood leucocytes and cerebral oedema. The pathogenesis of the disease is not known, nor is an effective vaccine available (reviewed in [10]). During dengue type 2 virus (DV) infection of mice a cytokine, called cytotoxic factor (CF), is produced by T cells of the spleen. CF is a highly potent protein molecule of 43–45 kD with an isoelectric point of pH 6.5. A sequence of 19 amino acids of the N-terminus of CF differ from

that of other known cytokines and the dengue virus-specific proteins (Gen Data Base, compared at Distributed Information Centre, Indian Institute of Science, Bangalore), thus indicating that it is a unique cytokine. Oligonucleotide probes derived from this sequence show the presence of mRNA for CF in the spleen cells of DV-infected mice ([11]; unpublished data). CF killed mainly H-2A-negative macrophages, T helper (Th) cells and megakaryocytes of lymphoid cells from a variety of animal species, including mice, by increasing influx of Ca^{2+} in them. CF induces H-2A-positive macrophages to produce another cytotoxin (CF2). CF/CF2 are capable of producing a number of pathological lesions in mice, which include reversible increase in vascular permeability and damage to the blood–brain barrier due to release of histamine. *In vitro* treatment of human blood leucocytes with CF decreases the E-rosetting capacity of T cells and the functions of monocytes, which are similar to the changes described in cases of DHF/DSS [12]. It would thus appear that CF is capable of reproducing the pathological lesions of DHF in mice [13–20]. The concept of pathogenesis-related proteins during viral infection of plants is well established [21]. It appears that CF is a pathogenesis-related protein, at least in mouse dengue infection.

Efforts have been made to neutralize the adverse effects of the cytokines by passive immunization, for example by administration of antibodies against TNF [1]. The pathological effects

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produced by CF/CF2 could be prevented by passive administration of the specific antibody in mice [16,17,19]. In the present study, an effort was made to investigate whether a vaccine could be developed for dengue using CF as antigen. We describe here our limited success in this regard.

MATERIALS AND METHODS

Mice

The study was carried out on inbred Swiss albino mice aged 2–3 months, obtained from the colony maintained in this Department.

Virus

DV, strain P 23085, obtained from the National Institute of Virology, Pune, India, was used in the form of infected mouse brain suspension. The titre of the virus was estimated in the brain tissue of mice following intracerebral (i.c.) inoculation of DV as described [16,22]. Briefly, serial 10-fold dilutions of the brain suspension were inoculated intracerebrally in groups of six mice each and the mortality was recorded. The virus titre was calculated by the method of Reed & Muench [23] and expressed as \log_{10} LD₅₀ for 30 μ l of the brain suspension. Normal mouse brain homogenate (NMB) was used as control in some experiments.

Preparation of the cytotoxic factor

CF was prepared from DV-infected moribund mouse spleen cells as described elsewhere [11] and its cytotoxic activity was assayed [24]. CF was purified with a Pharmacia low pressure liquid chromatography (LPLC) system using Sephacryl S-200 gel column, and was dried in a Speed Vac (Savant Instrument Company, New York, NY). The amount of protein was estimated by the technique of Lowry *et al.* [25]. The purity of CF was established by SDS-PAGE. Normal mouse spleen cell culture supernatant (NF) was similarly prepared and used as a control.

Immunization of mice

Mice were immunized with 5 μ g of purified CF protein emulsified in Freund's incomplete adjuvant (FIA; Sigma Chemical Co., St Louis, MO) subcutaneously on the dorsal aspect. The control mice were similarly given NF. At different intervals blood was collected from the eyes of mice, sera were separated and stored at -20°C .

Assay of blood-brain barrier permeability

The leakage of plasma proteins into the brain substance was measured by assay of plasma protein-bound Evans blue dye into the brain [16]. Briefly, mice were inoculated intravenously with 200 μ l of a 2% solution of Evans blue dye dissolved in PBS pH 7.2. After 1 h the mice were anaesthetized with halothene intraperitoneally and the brain was perfused with 5 ml PBS via the left heart ventricle. Whole brain was removed and a 10% (w/v) homogenate was prepared in PBS and centrifuged at 4°C at 6000 g for 15 min. The clear supernatant was screened for absorbance at 590 nm for the detection of protein-bound dye. The protein contents in the brain supernatant were calculated from the standard curve of bovine serum albumin (BSA) and expressed as μ g protein/ml [19]. The results were expressed as

permeability index (PI) of the blood-brain barrier [16], calculated as follows:

PI =

$$\frac{100 \times (\text{protein in DV-inoculated mice} - \text{background value})}{(\text{protein in NMB-inoculated control mice} - \text{background value})} - 100$$

Following challenge with CF the PI in immunized mice was compared with PI in normal control mice and expressed as per cent protection calculated as follows:

Per cent protection =

$$\frac{(\text{PI in immunized mice} - \text{PI in control mice})}{\text{PI in control mice}} \times 100$$

Assay of capillary permeability

Measurement of leakage of plasma protein-bound Evans blue dye from the vascular compartment into the peritoneal cavity of the mouse was used to estimate the integrity of the capillary permeability, as described previously [17]. Briefly, mice were inoculated intravenously with 100 μ l of Evans blue dye solution, followed 5 min later with CF intraperitoneally. After 30 min mice were anaesthetized and the peritoneal cavity was washed with 5 ml PBS. The lavage fluid was collected, filtered through glasswool column and made up to final volume of 10 ml. The optical density at 590 nm was determined with a spectrophotometer. The protein content of the fluid was calculated as μ g protein/ml. The results were expressed as PI, as described above. The control mice were inoculated with NF. The per cent protection in immunized mice was calculated as described above.

ELISA

The technique of Voller *et al.* [26] was used with some modifications to set up ELISA. Briefly, 100 μ l containing 3 ng of purified CF in PBS were added to the flat-bottomed ELISA plates (Titertek Immuno Assay Plates 77-173-05; Flow Labs, Zwanenburg, The Netherlands), and incubated overnight in a moist incubator at 37°C. The plate was washed three times with PBS containing 0.05% Tween 20 (PBS-T) and blocked with blocking buffer containing 1% milk protein (Lactogen-1 milk powder, Nestle India Ltd., New Delhi, India) in PBS for 1 h. The plate was washed again three times with PBS-T, and 100 μ l of 1:200 diluted serum from immunized or normal mice were added and the plate was incubated at 37°C for 1 h. After washing the plate, 100 μ l of protein A conjugated with horseradish peroxidase (HRP; a gift from the National Institute of Immunology, New Delhi, India) diluted 1:10 000 in dilution buffer (blocking buffer plus 0.5% Tween 20) were added and were incubated at 37°C for 1 h. The plate was washed and 100 μ l of the mixture of *o*-phenylenediamine and H₂O₂ in citrate buffer (0.02 M) pH 5.0 were added and incubated at room temperature for about 10 min. The reaction was stopped with 50 μ l of 2.5 N H₂SO₄ and the absorbance was read at 492 nm. Each serum sample was tested in duplicate and the mean absorbance in wells without CF was subtracted from the mean absorbance in wells with CF before analysis. The cut-off value for seropositivity was determined by adding 2 \times s.d. to the mean absorbance of normal mouse serum controls.

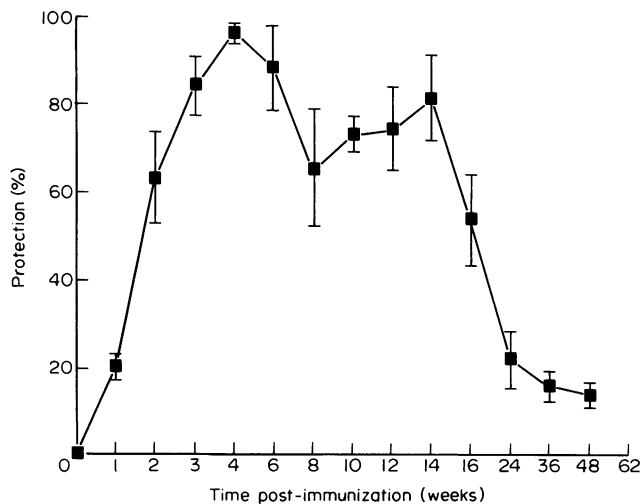


Fig. 1. Protection against cytotoxic factor (CF)-induced increase in vascular permeability of mice by specific active immunization. Groups of normal control and immunized mice at different periods were challenged with 3 μg of CF and the vascular permeability was assayed. The per cent protection was calculated as described in Materials and Methods. Each point represents mean value \pm s.d. from 10 to 12 mice.

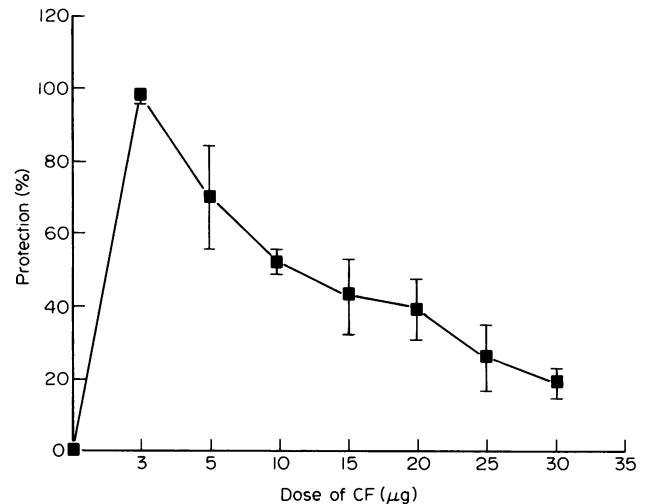


Fig. 2. Dose-dependent protection against cytotoxic factor (CF)-induced increase in vascular permeability of mice by specific active immunization. Groups of mice at week 4 of immunization and normal mice were challenged with various doses of CF and the vascular permeability was assayed and per cent protection calculated. Each point represents mean value \pm s.d. from 10 to 12 mice.

Assay of neutralization of the cytotoxicity of CF in vitro by sera of immunized mice

Sera obtained at different periods after immunization were doubly diluted and mixed with equal volume of CF and incubated at 37°C for 1 h. The target cells (2×10^6 normal mouse spleen cells) were then added to the mixture and incubated at 4°C for 1 h. Non-viable cells were then counted using the trypan blue dye exclusion method [24]. The control mice were similarly treated with normal mouse sera. Per cent neutralization of the cytotoxicity by immunized mice sera was calculated.

Plan of study

Mice immunized with CF were investigated at different periods from 1 to 48 weeks for their (i) capacity to withstand the challenge with CF itself or DV; (ii) presence of CF-specific antibodies; and (iii) the capacity of their sera to neutralize the cytotoxicity of CF on the target cells *in vitro*. The parameters used to measure the resistance to challenge with CF or DV were inhibition of the damage to (i) the blood-brain barrier and (ii) the vascular permeability in immunized mice compared with normal control mice. The data are presented as mean value \pm s.d. from 10 to 12 mice in each group. Student's *t*-test was used to analyse the data, and $P < 0.05$ was considered significant.

RESULTS

Effect of challenge of immunized mice with CF

CF-immunized mice were challenged with 3 μg of purified CF to assay the protective effect at different periods after immunization.

Effect on vascular permeability. The indicator of protective response was per cent decrease in CF-induced increase in capillary permeability in immunized mice compared with the normal control mice challenged with 3 μg of CF. The data summarized in Fig. 1 show that the extent of protection against

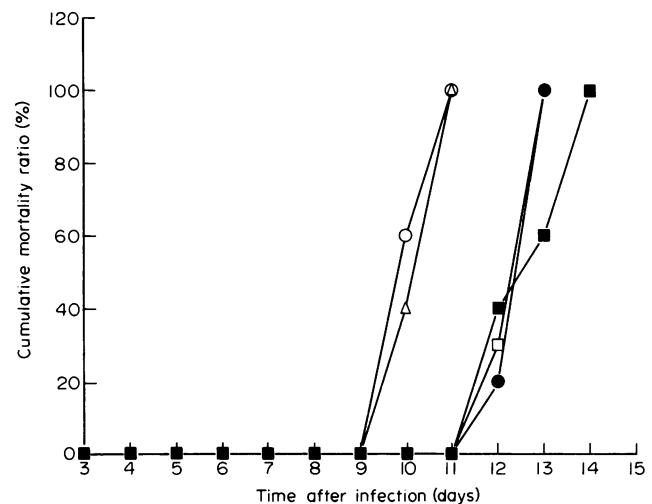


Fig. 3. Cumulative mortality of mice injected with 100 LD₅₀ of dengue type 2 virus (DV) intracerebrally (O), normal mice; □, at week 3; ●, at week 4; ■, at week 6; △, at week 20 after immunization. Each group consisted of 10 mice.

challenge with CF quickly increased with time after immunization, being 20 \pm 2% at week 1 to the peak of almost complete protection (96 \pm 3%) at week 4. The capacity to protect declined gradually up to week 14, when the protection varied between 65% and 80%. The protection was 21–39% during week 16 to week 48 after immunization.

Dose response of CF challenge. In another set of experiments the dose response of the CF challenge was investigated. For this, mice with peak protective response at week 4 after immunization were chosen. Groups of immunized and control normal

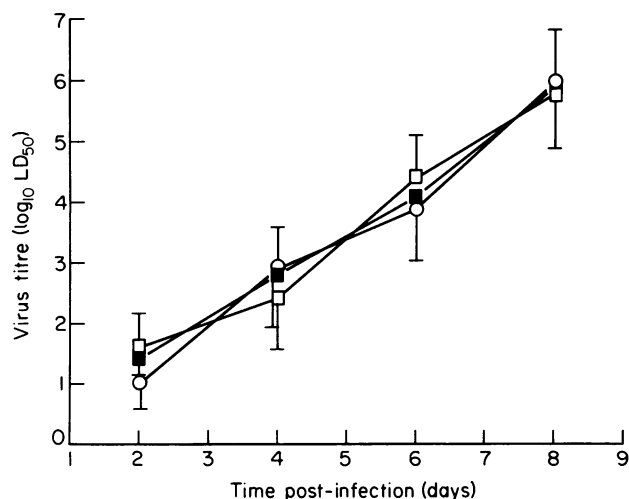


Fig. 4. Virus titres in the brain tissue of mice on different days after infection with 1000 LD₅₀ of dengue type 2 virus (DV) intracerebrally (■), normal mice; ○, at week 4; □, at week 10 after immunization. Each point represents mean value + s.d. from three mice.

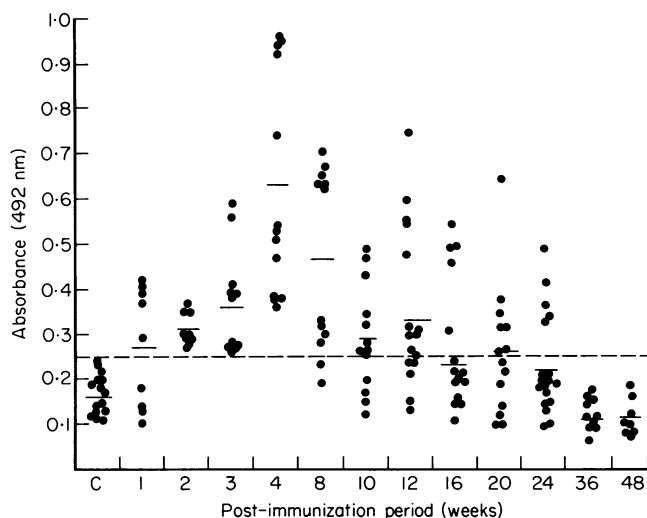


Fig. 5. Scatter diagram of ELISA seroactivity to cytotoxic factor (CF) in sera from normal healthy control mice and at different periods after active immunization with CF. Each point represents mean value of duplicate test on one serum sample. The horizontal bars indicate the mean absorbance for each group, and the dashed line the cut-off value for seropositivity. All points above the dashed line were considered seropositive.

mice were challenged with various doses of CF, and the per cent protection was calculated. The findings shown in Fig. 2 indicate that the capacity of immunized mice to resist the CF-induced increase in capillary permeability declined in a dose-dependent manner, being $96 \pm 3\%$ with $3 \mu\text{g}$ of CF and $19 \pm 4\%$ with $30 \mu\text{g}$ of CF.

Effect on blood-brain barrier. The effect of CF challenge on the blood-brain barrier was examined in a few groups of mice

which at weeks 4 and 26 after immunization were challenged with $3 \mu\text{g}$ of CF intravenously, and the permeability of the blood-brain barrier was assayed. It was observed that the protection at week 4 was $52 \pm 6\%$, while that at week 26 was $13 \pm 4\%$.

Effect of challenge of immunized mice with DV

The immunized and normal control mice were inoculated intracerebrally with 1000 LD₅₀ of DV, and various observations were made.

Sickness and mortality ratio. Following i.c. inoculation of normal mice with DV, the mice appeared healthy up to day 5 p.i. On day 6 p.i. arching of the back and ruffling of the fur occurred, which was followed by severe sickness with paralysis of limbs on days 8 and 9. All the mice died by day 11 p.i. The mean survival time was 10 ± 0.8 days. In contrast, the mice at 3, 4 and 6 weeks after immunization remained apparently healthy up to day 11 p.i., and no ruffling of fur or arching of back occurred. On days 12 or 13 they developed paralysis and died within the next 24 h. The mice at week 20 after immunization behaved like normal mice. The findings presented in Fig. 3 show the cumulative mortality rate in control and immunized mice. The curve has shifted significantly ($P < 0.001$) to the right with mice at 3, 4 and 6 weeks after immunization. The cumulative mortality ratio of mice at week 20 of immunization was similar to that of normal control mice.

Virus titre in the brain of mice. The immunized and normal mice were inoculated with 1000 LD₅₀ of DV. From each group three mice were killed 2, 4, 6 and 8 days p.i. and the brains were assayed for the virus titre. The data summarized in Fig. 4 show that the virus titres in the brains of immunized mice were similar to those of normal control mice.

Effect on the blood-brain barrier. Groups of mice at weeks 4 and 26 after immunization and the normal control mice were challenged with 1000 LD₅₀ of DV intracerebrally or intraperitoneally. The data obtained show that at week 4 after immunization protection to DV i.c. challenge with DV was $53 \pm 4\%$, and that to i.p. challenge was $86 \pm 7\%$. At week 26 after immunization it was $11 \pm 2\%$ and $17 \pm 4\%$, respectively.

Presence of anti-CF antibodies in immunized mice

Groups of mice immunized with CF were bled and the serum from individual mice was investigated for the presence of anti-CF antibodies at different periods.

ELISA test. At 1 week after immunization 56% of sera had anti-CF antibody titres above the cut-off value in the ELISA. The seropositivity was 100% during the weeks 2–8, and then declined gradually to 21% at week 24. At later periods none of the sera was positive (Fig. 5). The mean antibody titres gradually increased during the post-immunization period, reaching peak values at week 4 and then declining. This correlated with the extent of protection shown in Fig. 1.

Neutralization of the activity of CF by sera of the immunized mice. CF kills normal mouse spleen cells *in vitro* in 1 h at 4°C [24]. The protective effect of the sera from immunized mice on the cytotoxicity of CF was therefore investigated. The ability of the sera from immunized mice to neutralize the cytotoxicity of CF gradually increased with the time after immunization till week 4, persisted up to week 8, and then declined.

DISCUSSION

The significant finding of the present study is that active immunization by a cytokine (CF) protects mice against its adverse effects on subsequent challenge. The protection against the CF-induced increase in capillary permeability was complete at week 4 after immunization to a challenging dose of 3 µg/mouse of CF. A breakthrough in the protection occurred with higher doses of CF in a dose-dependent manner. The sera obtained from these mice had CF-specific antibodies as shown by ELISA and Western blot tests (results not shown), and had the capacity to neutralize the cytotoxic activity of CF *in vitro*. All these effects were dependent on the period after immunization.

On i.c. inoculation, DV replicates in the brain of mice, and produces encephalitis followed by paralysis of limbs and death. The virus spreads all over the body and can be detected in different organs [27,28]. DV lacks the ability to invade the central nervous system (CNS) when inoculated by peripheral routes (reviewed in [29]). Further, no apparent illness is produced by i.p. inoculation of DV in mice, but damage to the blood-brain barrier and increase in vascular permeability, mediated by CF, do occur [16,17]. The onset of paralysis was delayed, mean survival time was significantly prolonged ($P < 0.001$) and the clinical symptoms, like arching of the back and ruffling of the fur, were mostly absent in CF-immunized mice compared with the normal mice by lethal i.c. challenge with DV. The virus titres in the brain tissue were similar in the two groups. Similar prolonged mean survival times after a lethal challenge with viruses have been reported in T cell-deficient mice, such as athymic nude (*nu/nu*) mice [27] or those treated with anti-thymocyte serum [30]. In DV-infected mice the brain is damaged by replication of the virus and by a DV-induced T cell cytokine, the CF [16]. It has been shown that paralysis and death of mice occur due to replication of DV in the neuron cells, while damage to the blood-brain barrier, resulting in cerebral oedema and associated symptoms, is caused both by the virus and CF [16,29]. In the present study neither the virus titre nor the DV-induced paralysis and death could be prevented by CF immunization, but the latter was significantly delayed and the pathological lesions produced by challenging the mice with CF were prevented. On the other hand, in further experiments where the virus did not enter the brain (i.p. inoculation) the immunization provided specific protection ($86 \pm 7\%$) against damage to the blood-brain barrier on challenging with DV.

Several successful attempts have been made to immunize passively animals with anti-cytokine antibodies to neutralize its adverse effects. Beutler *et al.* [31] protected mice from the lethal effects of endotoxin by passive immunization against TNF. Development of cerebral oedema, haemorrhage and leakage of protein across the blood-brain barrier in cerebral malaria are prevented by antibodies against TNF, and a MoAb to *E. coli* [6]. Specific antisera protect mice against CF/CF2-induced increase in capillary permeability and breakdown of the blood-brain barrier [16-19].

However, it may be noted that not all antibodies against cytokines inhibit their function. Bendtzen *et al.* [32] have reviewed the role of autoantibodies to cytokines, and have suggested that anti-cytokine antibodies (neutralizing type) may produce 'cytokine deficiency', thus inhibiting their functions, while in some cases they (non-neutralizing type) contribute to a

positive therapeutic response by better targeting to the appropriate cells.

This initial success with active immunization using a cytokine has raised several questions: (i) what is the minimum dose of CF required?; (ii) how many booster doses are needed, and at what time?; (iii) what is the appropriate adjuvant for it?; and (iv) what are the side effects, if any? Studies are in progress in this direction.

Playfair *et al.* [3] have discussed the role of 'anti-parasite' versus 'anti-disease' vaccine in relation to malaria. They have cited evidence to show that soluble antigens (toxins) released from *Plasmodium falciparum* have properties of bacterial lipopolysaccharides and induce over-production of TNF which is responsible for the disease. Therefore, they have proposed the possibility of an 'anti-toxic vaccine' that prevents the serious pathological complications of the disease [3]. CF is a unique cytokine induced by DV which is responsible for the production of most of the pathological lesions which could be prevented by passive immunization [16,17,20], and also by active immunization as shown in the present study. Therefore, vaccine strategies directed at the cytokine (the primary cause) rather than the infective agents need serious consideration, especially when effective vaccines are not available for several agents.

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