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Effect of adjuvants on immunization with dengue virus-induced cytotoxic factor

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SUMMARY

Specific active immunization with dengue type 2 virus (DV)-induced cytokine, cytotoxic factor (CF), prevents CF-mediated pathology in mice. The present study was undertaken to determine the optimum dose of CF and the effect of different adjuvants on the immune response as assessed by the study of anti-CF antibody titre by ELISA and protection against increase in capillary permeability to challenging dose of 3 μg CF. The maximum protection of $94 \pm 4\%$ against increase in capillary permeability was observed at week 4 after immunization with 5 μg dose of CF mixed with Freund's incomplete adjuvant (FIA), which gradually decreased to $21 \pm 10\%$ on week 24. With a dose of 10 μg the protection obtained was $79 \pm 5\%$, but persisted for a longer time at a higher level. The response was poor with 1 μg dose of CF. The mean anti-CF antibody titres gradually decreased after reaching the peak at week 4 after immunization. Mice immunized with different adjuvants emulsified with 5 μg CF were challenged at different intervals with 3 μg CF. Maximum protection observed with CF + tetanus toxoid (TT) and 84/246 was about $93 \pm 2\%$ and $97 \pm 2\%$, while that with alhydrogel was $33 \pm 12\%$ and with bacille Calmette-Guérin (BCG) was $67 \pm 4\%$. At week 24 after immunization, however, the best response was obtained with 10 μg of adjuvant 84/246. Intracerebral challenge with 10 or 100 LD_{50} dose of dengue type 2 virus showed significantly prolonged mean survival time and delayed onset of signs of sickness in immunized mice compared with normal mice. The maximum survival time was with adjuvant 84/246 even at week 24. The findings thus show that the optimum dose of CF is 5 μg and the adjuvant of choice is 84/246.

Keywords adjuvant immunization cytokine dengue virus

INTRODUCTION

A cytokine called cytotoxic factor (CF) is produced during dengue virus infection of mice by T cells of the spleen, which is responsible for producing various pathological lesions that are seen in human dengue infection [1–8]. Thus CF appears to be a pathogenesis-related protein. Recently we have demonstrated the presence of a CF-like protein in the sera of human cases of dengue fever (DF), dengue haemorrhagic fever and shock syndrome (DHF/DSS) by ELISA and dot blot tests. Further, a CF-like protein has been purified from the sera of DHF cases by ion exchange and affinity chromatography (U. C. Chaturvedi *et al.*, unpublished data). Human peripheral blood $CD4^+$ T cells have been shown to produce CF on *in vitro* stimulation with dengue type 2 virus [9]. This has greatly enhanced the significance of CF in dengue pathogenesis. An effective vaccine for dengue virus infection is not yet available, so we have investigated the concept of 'anti-disease vaccine' by immunizing mice with CF mixed with Freund's incomplete adjuvant (FIA) and challenging them with CF. Almost complete protection against CF challenge was observed at week 4 of immunization which was dependent on the challenging dose of CF. This showed successful prevention of a cytokine-mediated pathology by specific active vaccination [10]. This initial success in 'anti-disease vaccination' has raised several questions. For example, what is the minimum dose of CF required for better results? Which adjuvant is best for immunization? The present study was planned to answer some of these questions.

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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

Dengue type 2 virus (DV), strain P23085, obtained from the National Institute of Virology (Pune, India) was used in the form of infected mouse brain suspension [11]. The virus titre was calculated by the method of Reed & Muench [12] and expressed as log 10 LD_{50} for 30 μl of the brain suspension. Normal brain homogenate (NMB) was used as control.

Preparation of the cytotoxic factor

CF was prepared from the spleen cells of DV-infected moribund mice. CF was purified from culture supernatant with Pharmacia low pressure liquid chromatography system using Sephacryl S-200 gel column [13,14], and dried in Speed Vac (Savant Instrument Inc., Farmingdale, NY). The amount of protein was estimated by the method of Lowry *et al.* [15]. The purity of CF was established by SDS–PAGE. Normal spleen cell culture supernatant (NF) was similarly prepared and used as a control.

Immunization of mice

Mice were immunized with different doses of purified CF emulsified in various adjuvants and injected subcutaneously on the dorsal aspect. The adjuvants used were FIA (Sigma Chemical Co., St Louis, MO), tetanus toxoid (TT; Behring Pharma Pvt. Ltd., Sathamrai A.P., India), alhydrogel (CDH Laboratory Reagents, New Delhi, India), bacille Calmette-Guérin (BCG; BCG Vaccine Laboatory, Madras, India), and a glycopeptide structurally related to muramyl dipeptide (MDP), N-acetyl normuramyl-L-N-methylalanyl-D-isoglutamine octalymide (Compound no. 84/246) was synthesized at the Central Drug Research Institute (Lucknow, India) [16]. Control mice were similarly treated with adjuvant alone or mixed with NF. At different intervals blood was collected from the eyes of mice, sera were separated and stored at −20°C.

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 capillary permeability as described previously [5]. Briefly, mice were inoculated intravenously with 100μl 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 saline. The lavage fluid was collected and filtered through glass wool column and made up to final volume 10 ml. Optical density at 590 nm was determined with a spectrophotometer. The protein contents of the fluid were calculated as μg protein/ml. The results were expressed as permeability index (PI). Control mice were inoculated with NF. The per cent protection in immunized mice was calculated as follows:

$$PI = \frac{100 \times (\text{protein in CF inoculated mice} - \text{background value})}{(\text{protein in NF inoculated mice} - \text{background value}) - 100}$$

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

ELISA

The technique of Voller *et al.* [17] was used with some modifications to set up ELISA [10]. Briefly, 3 ng of purified CF in 100 μl PBS were added to the flat-bottomed ELISA plate (Titertek Immuno Assay plates 77-173-05; Flow Labs, Zwanenburg, The Netherlands), incubated overnight at 37°C in humidified atmosphere. 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 μl of 1 : 200 diluted serum from immunized or normal mice sera for control were added and the plate was incubated at 37°C for 1 h. After washing the plate, 100 μl of protein A conjugated with horseradish peroxidase (HRP; a gift from the National Institute of Immunology, New Delhi, India) (1 : 10 000) were added and incubated at 37°C for 1 h. The plate was washed and a 100 μl mixture of *o*-phenylenediamine and H_2O_2 in citrate buffer (0·02 M) pH 5·0 was added, and 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 × s.d. to mean absorbance of normal mouse serum control.

RESULTS

Immunization with different doses of CF

Mice were immunized with different doses of CF emulsified in FIA. The immunized and the control mice were challenged with 3 μg of purified CF and capillary permeability was assayed to find the protective effect at different periods after immunization. Indication of the protective response was per cent decrease in CF-induced increase in capillary permeability in immunized mice compared with normal control mice. The data summarized in Fig. 1 show that protection against challenge with CF was maximum (94 ± 4%) at week 4 with an immunizing dose of 5 μg CF which gradually decreased to 21 ± 10% on week 24. With the immunizing doses of 1 or 10 μg of CF the

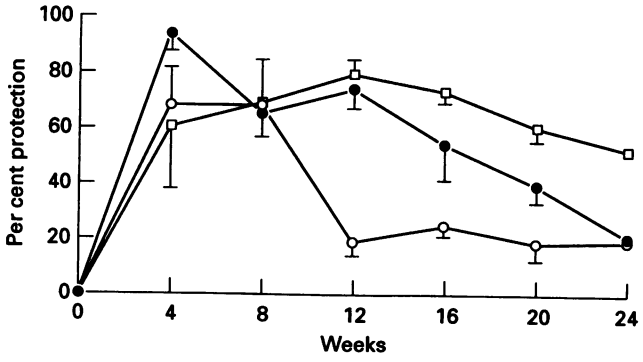

Fig. 1. Effect of immunizing dose of cytoxic factor (CF) on the capacity to protect mice against increase in capillary permeability by challenge with 3 μg of CF at different periods. Mice were immunized with 1 μg (○), 5 μg (●), or 10 μg (□) CF emulsified in Freund's incomplete adjuvant (FIA). Control mice were treated with normal spleen cell culture supernatant (NF) mixed with FIA, while those for background values were treated with FIA alone. Each point represents mean ± s.d. from 8–10 mice.

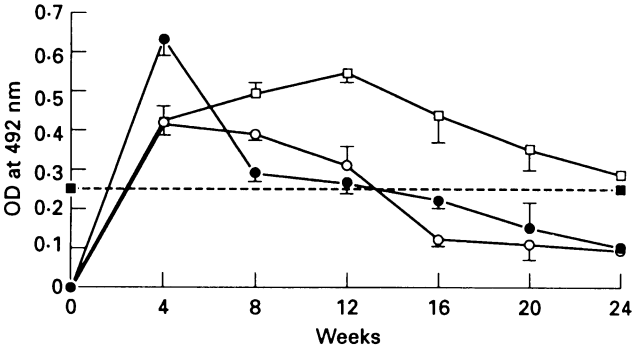

Fig. 2. Anti-cytotoxic factor (CF) antibody titre in mice immunized with different doses of CF. Sera collected from individual mice of Fig. 1 were tested by ELISA. Mice immunized with 1 μg (○), 5 μg (●), or 10 μg (□) CF. Mean OD + 2 s.d. on sera from control mice is shown as dotted horizontal line.

protection obtained was less than 79 ± 5%. Mice immunized with different doses of CF were bled at different periods and the sera from individual mice were assayed for the presence of anti-CF antibodies by ELISA. The findings summarized in Fig. 2 show that with an immunizing dose of 1 μg, mean antibody titre gradually decreased after reaching a peak at week 4 of immunization. With 10 μg of immunization dose seropositivity was 100%, and higher antibody titres persisted up to week 24 of observation. The mean antibody titre gradually decreased after week 12 with 1 and 5 μg immunization dose.

Effect of adjuvants on immunogenicity of CF

Groups of mice were immunized with 5 μg CF mixed with different adjuvants to study the efficacy of the adjuvant. Control mice were given only the adjuvant. Protection against an increase in capillary permeability to a challenge with 3 μg of CF was studied as described earlier. The results observed after week 4 of immunization showed maximum protection of 97 ± 2% in mice immunized with CF + 84/246. Mice immunized with CF + TT showed 93 ± 6% and with

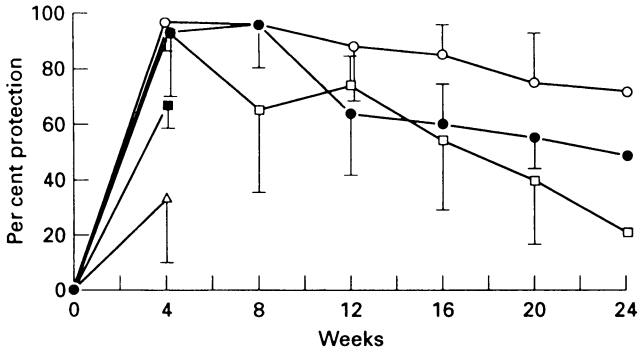

Fig. 3. Protective effect of immunization of mice with 5 μg of cytotoxic factor (CF) mixed with different adjuvants against increased capillary permeability to a challenge with 3 μg of CF. Control mice were given normal spleen cell culture supernatant (NF) mixed with different adjuvants and those treated with the adjuvant alone yielded background values. Each point represents mean ± s.d. from 10 mice. Freund's incomplete adjuvant (FIA) (□), 84/246 (○), tetanus toxoid (TT) (●), bacille Calmette–Guérin (BCG) (■), and alhydrogel (△).

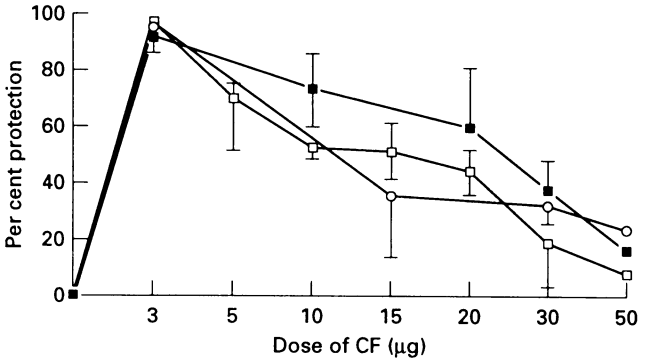

Fig. 4. Dose response to challenge with various doses of cytotoxic factor (CF). Mice immunized with 5 μg of CF mixed with tetanus toxoid (TT) (○), Freund's incomplete adjuvant (FIA) (□) or 84/246 (■) were challenged with various doses of CF and protection against increase in capillary permeability was recorded. The controls were as described above. Each point represents mean ± s.d. from 10–12 mice.

CF + FIA 94 ± 4% protection, while that with alhydrogel was 33 ± 12% and with BCG 67 ± 4%. At week 24 of immunization, however, the best response (72 ± 6%) was obtained with adjuvant 84/246 (Fig. 3).

In another set of experiments the dose response of CF challenge was investigated in mice immunized by CF mixed with TT or FIA or 84/246. For this, mice were taken at week 4 after immunization. Groups of immunized and control mice were challenged with various doses of CF and per cent protection was calculated. The data presented in Fig. 4 show that the capacity of immunized mice to resist the CF-induced increase in capillary permeability declined in a dose-dependent manner with all the three adjuvants, but the decline was least with 84/246.

An attempt was made to find the optimum dose of 84/246 adjuvant. Mice were immunized with 5 μg of CF mixed with different doses of adjuvant 84/246 and challenged with 3 μg of purified CF at week 4 after immunization, and capillary permeability was assayed. The data summarized in Fig. 5 show that 10 μg dose of the adjuvant is best, as up to week 24 the protection level was 72 ± 6%. With higher doses of the adjuvant the background values were very high (data not shown).

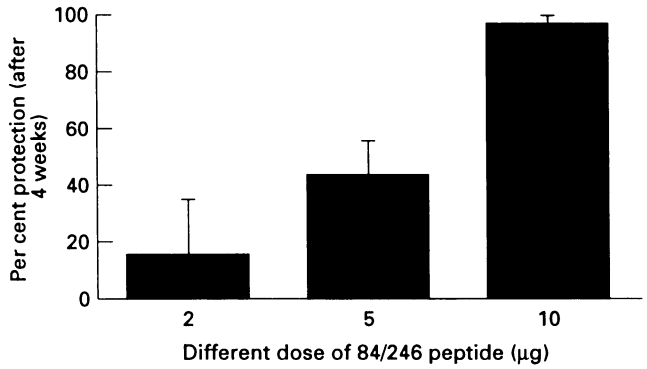

Fig. 5. Effect of various immunizing doses of 84/246 mixed with 5 μg of cytotoxic factor (CF) on the capacity to protect against increase in capillary permeability to challenge with 3 μg CF at week 4. Each group consisted of 10 mice.

Effect of challenge of immunized mice with DV

Mice immunized with different adjuvants mixed with 5 μg of CF and normal control mice were inoculated intracerebrally with 10 or 100 LD_{50} of DV, and the sickness and mortality ratio were recorded over a period of 21 days. The normal mice inoculated with DV (100 LD_{50}) remained healthy up to day 5 post-infection (p.i.), and on day 6 arching of the back and ruffling of the fur occurred. A severe sickness and paralysis of the limbs occurred on days 8–9, and all mice died by day 12 p.i. The events shifted to the right by 1 day in mice given 10 LD_{50} of DV. The mean survival times were $10{\cdot}2 \pm 0{\cdot}4$ and $11{\cdot}4 \pm 0{\cdot}5$ days with 100 and 10 LD_{50} of DV, respectively (Fig. 6a). At week 4 after immunization the mean survival time was 15–16 days ($P =< 0{\cdot}001$) with 10 LD_{50} and 12–16·4 days ($P > 0{\cdot}05$ to $< 0{\cdot}001$) with 100 LD_{50} of the virus in different groups of mice. At 24 weeks after immunization the mean survival time was prolonged only in mice given 84/246 adjuvant, being $19 \pm 1{\cdot}5$ days with 10 LD_{50} and $16 \pm 0{\cdot}8$ days with 100 LD_{50} of DV (Fig. 6a,b). The immunized mice did not develop the milder signs of illness; they developed paralysis and died within 24 h.

DISCUSSION

The main finding of this series of experiments is that 84/246 is the best adjuvant for immunization of mice. A complete protection ($97 \pm 2\%$) against increase in capillary permeability to a challenging dose of 3 μg/mouse was observed at week 4 after immunization. The peak protection obtained with 10 μg immunizing dose was $79 \pm 5\%$, but persisted at a higher level for a longer period. The route of exposure, *in vivo* persistence, dose, frequency and physical properties of antigen have an influence on the type and length of immunity. Native T cells proliferate during their encounter with antigens, and an expanded pool of memory T cells develops and persists even for a life time [18]. For an optimum immune response, booster doses of an antigen are required. Further, the adjuvants also influence the immune response to an antigen. FIA and *Corynebacterium parvum* enhance significantly DTH response, and saponin enhances humoral immune response [19]. Aluminium-containing compounds such as hydroxides and phosphates are approved for clinical use, but they are not active with all immunogens and stimulate only humoral responses [20]. The protection observed at week 4 was above 90% by using TT or 84/246 as adjuvant, whereas with BCG and alhydrogel it was $67 \pm 4\%$ and $33 \pm 12\%$, respectively. At week 24 protection continued to be higher ($72 \pm 6\%$) only with adjuvant 84/246. Challenge of immunized mice with DV showed significantly prolonged mean survival time, absence of signs of illness like arching of the back and ruffling of the fur, and delayed onset of paralysis compared with normal mice. Peak response was obtained with adjuvant 84/246, which increased the mean survival time to 16–19 days in different groups of mice. These findings support our earlier observations [10]. Interferon-gamma (IFN-γ) selectively augments the production of IgG2a antibodies in mice [21]. The depot type adjuvants (FIA, Freund's complete adjuvant (FCA) and alhydrogel) and MDP are effective in promoting the immune response to bovine serum albumin (BSA) [19]. The immunostimulant activity of 84/246 is comparable to that of MDP [16]. Immunoadjuvants are a class of compounds with a large variety of conformations and act via different pathways. Their biological properties depend upon their capacity to activate selectively Th1 or Th2 cells that control the major features of specific immune response [22]. It has been shown that FCA activate Th1 cells and alhydrogel Th2 cells of mice, but it may also be relevant to humans [23]. The basis of adjuvanticity of microbial components could be their recognition by phylogenetically ancient receptors present on accessory cells. This recognition induces them to produce cytokines which selectively stimulate Th1 or Th2 cells [24].

A question that needs to be addressed is whether such a vaccine could ever find practical use, because it is based on immunization against a self protein. Presence of autoantibodies to a number of cytokines has been described and their role discussed. Anti-cytokine autoantibodies are known to block the activity of the cytokines, and are found in the serum for IL-1α, IL-2, IL-4, IL-6, IL-8, IL-10, tumour necrosis factor-alpha (TNF-α) and IFN-α. Small complexes between such antibodies and cytokines do not activate complement if IgG4 antibodies are involved, and do not precipitate *in vivo*, but are active as inflammatory complexes [25]. Bendtzen *et al.* [26] have proposed that a major role of anti-cyctokine autoantibodies is to facilitate, rather than neutralize, functions of cytokines in the body by acting as specific physiological carriers and regulators of cytokines. For effective immunization it is essential that such

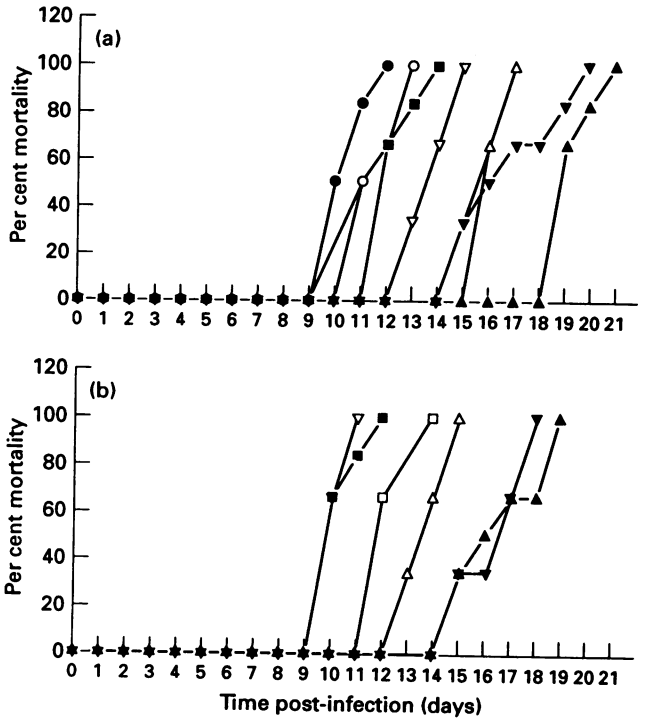

Fig. 6. Cumulative mortality ratio of mice injected with dengue type 2 virus (DV) intracerebrally. Normal mice give 10 LD_{50} (○) or 100 LD_{50} (●) of DV. Mice immunized with cytotoxic factor (CF) mixed with Freund's incomplete adjuvant (FIA) (□), tetanus toxoid (TT) (△), or 84/246 (▲) were challenged with 10 LD_{50} of DV, while second set of mice immunized with CF mixed with FIA (■), TT (▽) or 84/246 (▼) were challenged with 100 LD_{50} of DV intracerebrally. (a) At 4 weeks after immunization. (b) At 24 weeks after immunization. Each group consisted of six mice.

epitopes of cytokines are used which generate neutralizing antibody only.

Ideally an adjuvant should induce both high antibody levels and strong cell-mediated immunity and should be safe and comfortable. FCA is unacceptable for human use, while FIA is no longer used, although there is no evidence that it produces any adverse effects. Alum remains an important adjuvant for administration with preventive vaccines and has recently been used with synthetic malaria vaccine. In some of the clinical trials cytokines like interferons and IL-2 have been tried as adjuvant. CF is an unique cytokine induced by DV which is responsible for the production of most of the pathological lesions which could be prevented by active immunization [10]. The effect of the vaccine could be enhanced by using booster doses and appropriate adjuvants. The effect of booster doses remains to be investigated.

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