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Macrophage functions during dengue virus infection: antigenic stimulation of B cells

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SUMMARY

This study was undertaken to investigate the function of dengue type 2 virus (DV)-infected mouse peritoneal macrophages (Mϕ) regarding the antigenic stimulation of B lymphocytes of the spleen. It was observed that a variable proportion of Mϕ show DV-specific immunofluorescent antigen, which depended upon the route of administration of the virus, being higher in i.p.-inoculated mice and *in vitro*-infected Mϕ monolayers. The DV-infected Mϕ presented the DV antigen to B cells *in vitro* and *in vivo*, leading to their clonal expansion as shown by counting the virus-specific IgM antibody plaque-forming cells (PFC). The PFC response depended upon the number of DV-infected Mϕ. The antigen was presented equally well both by I-A-negative and I-A-positive Mϕ. Superimposition of a heterologous antigen (Coxsackie B4 virus) in a Mackaness type of experiment depressed the capacity of Mϕ to present both the homologous as well as heterologous antigen.

INTRODUCTION

Cells of the monocyte–macrophage system are a major component in the host defence against viral infections, and can act by intrinsic or extrinsic mechanisms. Macrophages (Mϕ) are also known to participate in immunopathogenetic mechanisms triggered by viruses and may have variable relationships with them: Mϕ inactivate some of the viruses or act as a passive carrier for them; on the other hand, they are permissive to some of the viruses (Denman & Pinder, 1974). The optimum immune response to an antigen depends on the co-operation between Mϕ and T and B cells. Perturbation of immune responses can occur due to damage of Mϕ by the replicating virus.

Mϕ are the principal cells to replicate dengue viruses in different species including mice (Hotta *et al.*, 1981; Chaturvedi, Nagar & Mathur, 1983). In dengue type 2 virus (DV)-infected mice, a state of immunosuppression develops which is characterized by the absence of cell-mediated immune responses (CMI) to the infecting virus, and depression of humoral response and CMI to heterologous antigens (Chaturvedi, Tandon & Mathur, 1977; Chaturvedi *et al.*, 1978b, 1981b). This is associated with splenic atrophy, reduced T-cell counts in the spleen and of Mϕ in the spleen and peritoneal cavity; the proportion of Mϕ is decreased up to 75% in the peritoneal cavity (Tandon, Chaturvedi & Mathur, 1979; Chaturvedi *et al.*, 1983). The functions like phagocytosis, attachment and migration of Mϕ are markedly depressed in such mice (Gulati, Chaturvedi & Mathur, 1982; Chaturvedi *et al.*, 1983). The adverse effects of DV on the immune system appear to mediated by a DV-induced cytotoxic pathway which involves the production of a cytotoxic factor (CF) by the T lymphocytes of the spleen, and which induces a subpopulation of Mϕ to produce another cytotoxin (CF_2). Both CF and CF_2 kill or damage the Mϕ and some of the T lymphocytes to produce non-specific immunosuppression (reviewed by Chaturvedi, 1986).

A major function of Mϕ is the uptake, processing and presentation of antigen in a highly immunogenical form to T and B lymphocytes to generate an optimum response. DV induces the depression of Mϕ functions and immunosuppression; therefore, it was considered worthwhile to investigate the functions of DV-infected Mϕ as regards the antigen presentation to B cells. It was observed that DV-infected Mϕ can present the homologous antigen to B cells, but when a heterologous antigen is superimposed the presentation of both the antigens is depressed.

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MATERIALS AND METHODS

Animals

Adult inbred Swiss albino mice (3–4 months old) obtained from the colony maintained in the Department were used.

Viruses

Dengue type 2 virus (DV), strain P23085, was used in the form of adult mouse brain suspension in a dose of 1000 LD_{50} (Chaturvedi *et al.*, 1977). The standard strain of Coxsackie B_4 virus (CoxBV) obtained from the Haffkine Institute, Bombay was used in the dose of 1000 $TCID_{50}$ (Chaturvedi, Tandon & Mathur, 1978a; Tandon *et al.*, 1978).

Preparation of Mϕ monolayers

Mϕ monolayers were prepared from the peritoneal cells collected by inoculation of 5 ml heparinized Eagle's minimum essential medium (MEM)/mouse intraperitoneally (i.p.). The aspirated cells were layered on glass petri-dishes (2×10^6 cells), or on 20×20 mm glass coverslips placed in Petri-dishes, and incubated for 2 hr at 37° in 5% CO_2. Non-adherent cells were removed by washing three times with Hanks' balanced salt solution (HBSS). The glass-adherent cell sheet contained more than 95% phagocytic cells, as shown by the ingestion of latex particles (Chaturvedi, Shukla & Mathur, 1982) and have been considered as Mϕ monolayers in the present study.

Preparation of spleen cell cultures

Normal mouse spleen cells were teased out gently in chilled MEM containing 10% fetal calf serum (FCS) (Armour Pharmaceutical Co., U.K.). A single cell suspension was prepared and viable nucleated cells were counted using trypan blue dye exclusion (Chaturvedi *et al.*, 1978a). The spleen cells suspended in HEPES-MEM containing 10% FCS and 5×10^{-5} M 2-mercaptoethanol were cultured in 5-cm glass petri-dishes, each containing 4 ml cell suspension (5×10^6 cells/ml) and were incubated at 37° in presence of 5% CO_2.

Preparation of cytotoxic factor (CF)

DV induces production of CF by T cells in the mouse spleen which kills nearly 70% I-A-negative and spares I-A-positive Mϕ (Chaturvedi, Bhargava & Mathur, 1980a; Chaturvedi *et al.*, 1981a, 1981b, 1987a). CF was prepared from the homogenate of DV-infected mouse spleen (Chaturvedi, Dalakoti & Mathur, 1980b; Gulati *et al.*, 1983).

Depletion of I-A-positive Mϕ

The Mϕ monolayers were depleted of I-A-positive cells by treatment with anti-I-A^k antibody (kindly provided by Dr M. Feldmann, The Charing Cross Sunley Research Centre, London) and complement as described elsewhere (Shukla, Dalakoti & Chaturvedi, 1982; Chaturvedi *et al.*, 1987a). The depletion of I-A-positive cells was confirmed by an indirect immunofluorescent technique, as described elsewhere (Chaturvedi *et al.*, 1987a). The cells were washed three times with HBSS and used in further experiments.

Fluorescent antibody study of DV antigen

DV infection in Mϕ was shown by demonstration of the virus-specific antigen in the cells using the fluorescent antibody technique (FAT). Mϕ monolayers were fixed in chilled acetone, then treated with virus-specific antibody and prepared for indirect fluorescent antibody study (Gardner & McQuillin, 1981). The cells were examined in a Leitz Dialux 20 fluorescence microscope (Leitz, FRG).

Assay for antigenic stimulation

The antigenic stimulation function of Mϕ was assayed by counting antigen-specific IgM antibody plaque-forming cells (PFC) in spleen cells by the localized haemolysis gel technique of Jerne & Nordin (1963) as described elsewhere (Tandon & Chaturvedi, 1977; Chaturvedi *et al.*, 1977). This was done *in vitro* in spleen cell cultures and *in vivo* in mice. The DV-infected Mϕ monolayers were washed and co-cultured with normal mouse spleen cells (20×10^6 cells/petri-dish) for different periods. The PFC responses against DV peaks on Day 4 (Shukla & Chaturvedi, 1981); therefore, the spleen cells were collected on that day and processed for PFC count. In *in vivo* experiments, normal mice were inoculated i.p. with 1×10^6 DV-infected Mϕ followed by counting of DV-specific IgM PFC in the spleen cells on Day 7 (Tandon & Chaturvedi, 1977). The mean values ± SD of the data obtained from eight to 12 mice in repeated experiments have been presented after deducting background PFC, and have been analysed using the Student's *t*-test for *P* value. A *P* value of more than 0·05 was considered insignificant.

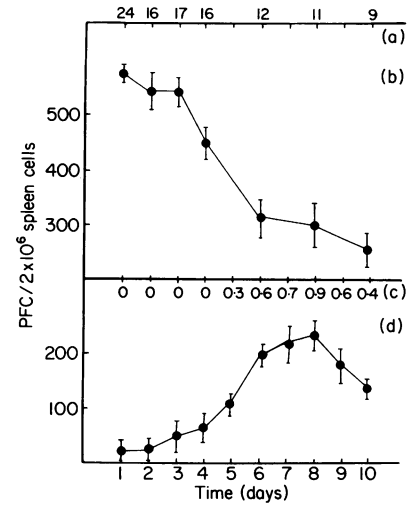

Figure 1. Induction of the virus-specific IgM PFC by the DV-infected peritoneal Mϕ obtained from mice given the virus by i.p. (b) or i.c. (d) route. Virus-antigen-positive immunofluorescence cells per 10^2 Mϕ in mice given DV by i.p. (a) or i.c. (c) routes.

RESULTS

Antigenic stimulation by Mϕ from mice infected intracerebrally (i.c.)

Mice inoculated i.c. with DV were killed in groups from Days 1 to 10 and their peritoneal cells collected. After washing, 1,000,000 cells were inoculated i.p. in normal mice and DV-specific IgM PFC were counted on the 7th day. For background PFC, uninfected peritoneal cells were similarly injected. Cells were also prepared from each mouse for FAT for DV-specific antigen. The findings presented in Fig. 1d show a negligible PFC response by Mϕ obtained up to Day 4 p.i. It gradually increased to peak PFC response on the 6th day and then declined. Virus-specific antigen was present in Mϕ from the Day 5 p.i. with the maximum number of cells being positive (0·9%) on Day 8 (Fig. 1c). Thus the findings show that DV-infected Mϕ can present the virus antigen to B cells.

Antigenic stimulation by Mϕ from mice infected i.p.

Mice given DV i.p. were killed in groups at different periods and their peritoneal cells were collected. From one aliquot, cells were prepared for FAT. After washing three times 1,000,000 cells from the other aliquot were inoculated i.p. in normal mouse and on the 7th day DV-specific IgM PFC were counted in the spleen cells. For background PFC, mice were similarly inoculated with

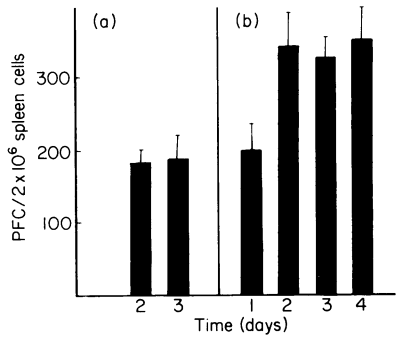

Figure 2. Induction of the virus-specific IgM PFC by peritoneal Mϕ infected with DV *in vitro*. (a) The spleen cells remained in contact with infected Mϕ for 24 hr and were then cultured separately; or (b) remained in contact for different periods.

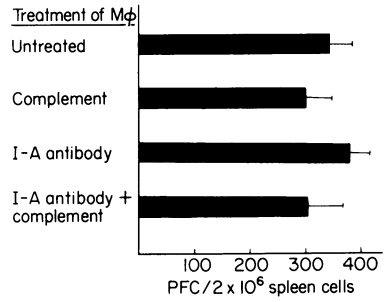

Figure 3. Induction of the virus-specific IgM PFC by Mϕ pretreated with anti-I-A antibody and then infected with DV *in vitro*.

normal mouse peritoneal cells. The data presented in Fig. 1b show a peak PFC response in mice given peritoneal Mϕ collected 24 hr after infection, when it was $580 \pm 38/2 \times 10^6$ spleen cells ($P < 0{\cdot}001$). The count gradually declined with the increasing period of infection. DV-specific antigen was present in a maximum number of Mϕ obtained 24 hr after infection. The number of the virus infected Mϕ gradually declined with $9 \pm 2\%$ Mϕ being positive on Day 10 p.i. (Fig. 1a).

Antigenic stimulation by Mϕ infected in *in vitro*

Mϕ monolayers were inoculated with DV and incubated at 37° for 1 hr. After washing three times, the Mϕ monolayer was covered with normal mouse spleen cells. In one set of experiments the cells were separated after 24-hr contact with DV-infected Mϕ and cultured separately. In the other set of experiments, the spleen cells were allowed to remain in contact with the DV-infected Mϕ for variable periods and then DV-specific IgM PFC were counted in the spleen cells as shown in Fig. 2. For background PFC, the spleen cells were similarly treated with uninfected Mϕ. At each period Mϕ monolayers were prepared for FAT, and virus-specific antigen was studied. It was observed that when spleen cells were separated from DV-infected Mϕ monolayers and then cultured, the PFC count was low, being 179 ± 22 at Day 2 and 187 ± 31 at Day 3 (Fig. 2a). In contrast, when the spleen cells remained in contact with the

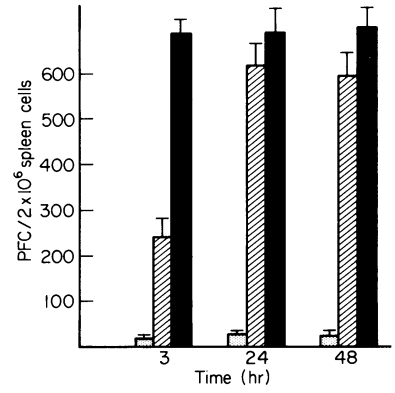

Figure 4. Induction of the virus-specific IgM PFC by Mϕ infected with DV at different periods after pretreatment with CF (▨) or without it (■). Normal mouse Mϕ were (▦) used for background PFC.

virus-infected Mϕ the PFC count was significantly higher, being 326 ± 27 ($P < 0{\cdot}001$) to 341 ± 37 ($P < 0{\cdot}001$) between Days 2 and 4 (Fig. 2b).

Following DV infection *in vitro*, the virus-specific antigen was present in $40 \pm 8\%$ Mϕ at 24 hr and in $54 \pm 9\%$, being the maximum number, after 72 hr. Therefore, in another experiment the Mϕ monolayers were infected with DV and layered with normal mouse spleen cells after 72 hr. IgM PFC were counted on Day 4 after remaining in contact with the Mϕ. The PFC counts in this experiment were similar to those in the above experiment.

Effect of depletion of I-A-positive Mϕ on antigenic stimulation

Mϕ monolayers pretreated with anti-I-A^k antibody and complement were washed three times and inoculated with DV. After 1 hr the Mϕ monolayers were washed and covered with normal mouse spleen cells and cultured for 4 days and then DV-specific IgM PFC were counted. Untreated Mϕ monolayers, or those treated with the antibody or complement alone, were included for control. Variously treated Mϕ monolayers were also studied for the presence of virus antigen by FAT after 24 hr of the inoculation of the virus. The findings presented in Fig. 3 show that the PFC count in the sets with anti-I-A antibody and complement-treated Mϕ was 302 ± 60, while in that without any treatment it was 338 ± 43 ($P > 0{\cdot}10$). FAT showed presence of DV antigen in $38 \pm 6\%$ untreated Mϕ and $28 \pm 4\%$ Mϕ treated with anti-I-A antibody and complement.

Antigenic stimulation by Mϕ of mice treated with cytotoxic factor

DV-induced CF selectively kills I-A-negative Mϕ and spares I-A-positive Mϕ (Chaturvedi *et al.*, 1987a); therefore, CF was used to study the effect of depletion of I-A-negative Mϕ on the antigen presentation. Mice pretreated with 0·25 ml (1:30 dilution) of CF i.p. were given DV i.p. at 3, 24 and 48 hr (Chaturvedi *et al.*, 1987a). The virus was allowed to act for 1 hr and then the peritoneal cells were collected, washed three times and 1,000,000 cells inoculated i.p. per normal mouse. On the 7th day, DV-specific IgM PFC were counted in the spleen cells of recipient mice. For background PFC, mice were similarly inoculated with peritoneal cells from normal mice or those

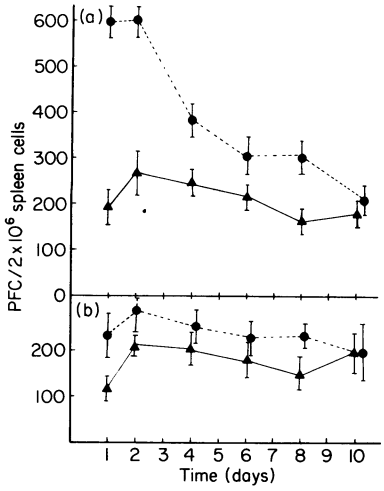

Figure 5. Induction of the virus-specific IgM PFC by DV-infected Mϕ infected with CoxBV at different periods. (a) DV-specific PFC induced by Mϕ infected by DV alone (●) or by DV plus CoxBV s(▲). (b) CoxBV-specific PFC induced by Mϕ infected with CoxBV alone (●) or by DV plus CoxBV (▲).

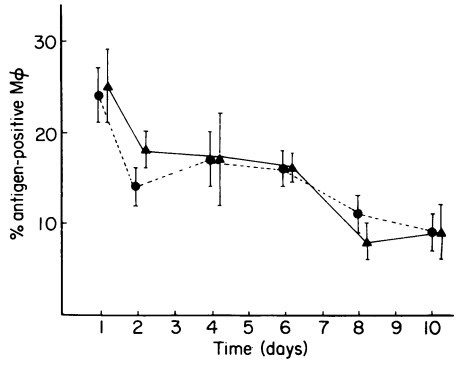

Figure 6. Demonstration of the DV antigen-positive Mϕ in mice given DV i.p. alone (●) or by DV plus CoxBV (▲) given at different periods after DV.

treated with CF only. The findings were compared with the PFC in mice given Mϕ obtained from mice treated with DV i.p. only. The data presented in Fig. 4 show that Mϕ at 3 hr after treatment with CF induced 240±42 PFC as compared to 678±29 PFC by untreated Mϕ infected by DV ($P<0{\cdot}001$). Thus the reduction in PFC was 65%. At later periods, greater numbers of PFC were produced by the CF-treated group and were 11–15% less than the controls. The non-viable cell counts in the peritoneal fluid were 51±3, 20±5 and 6±2% at 3, 24 and 48 hr, respectively, after the CF treatment.

Stimulation with heterologous antigen by DV-infected Mϕ

A Mackaness type of experiment was done to investigate the capacity of DV-infected Mϕ to present an unrelated antigen to B cells. Mice given DV i.p. were given CoxBV i.p. at the same time or at different periods. After 24 hr of CoxBV inoculation the peritoneal cells were collected, washed three times and 1,000,000, of them injected i.p. in normal mice. On the 7th day, DV-specific and CoxBV-specific IgM PFC were counted in the spleen. The control mice received Mϕ from uninfected mice or those infected with either DV or CoxBV. Mϕ of mice given DV plus CoxBV or DV alone were screened for DV-specific antigen by FAT.

The data of the DV-specific PFC presented in Fig. 5a show a sharp decline in the capacity of Mϕ to present DV antigen when CoxBV infection was superimposed at early periods. The DV-infected Mϕ obtained at 24 hr induced 598±38 PFC, which was reduced to 190±37 ($P<0{\cdot}001$) when CoxBV was also added. The PFC count in mice given both the viruses was between 165±27 to 268±49 throughout the period of study. The findings of CoxBV-specific PFC presented in Fig. 5b show that the number of PFC produced by Mϕ infected by CoxBV alone were much more ($P<0{\cdot}001$–$0{\cdot}025$) than those by DV plus CoxBV infected. Thus the capacity to present both homologous and heterologous antigen was reduced, being very marked for the former.

An effort was made to investigate the effect of CoxBV infection on the proportion of Mϕ manifesting DV antigen. The data presented in Fig. 6 show that the proportion of Mϕ showing DV antigen by FAT was similar throughout the period of study in the two groups, one receiving DV and the other DV plus CoxBV ($P>0{\cdot}50$).

DISCUSSION

The findings of the present study demonstrate that DV-infected Mϕ present the virus-antigen to B lymphocytes *in vivo* and *in vitro*, resulting in clonal expansion of antigen-specific cells. Mϕ monocytes are the principal cells to replicate DV, as shown by *in vivo* and *in vitro* studies (Halstead, 1981; Hotta *et al.*, 1981, 1983; Chaturvedi *et al.*, 1983). In the present study the infection of the Mϕ was assessed by the demonstration of DV-specific immunofluorescent antigen in the peritoneal Mϕ of mice given DV by i.c. or i.p. route or by infection *in vitro*. A significantly larger number of Mϕ were infected at 24 hr by giving the virus by i.p. route (24±4%, $P<0{\cdot}001$) or *in vitro* (40±6%, $P<0{\cdot}001$) in contrast to the peak of 0·9% on Day 8 of the virus by i.c. route. The induction of PFC was related to the proportion of the infected Mϕ, thus the higher PFC response was induced by Mϕ infected *in vitro* or by the i.p. route, compared to those by i.c. route. The period of peak PFC response varied in different groups depending upon the optimum number of Mϕ showing the virus antigen. Thus, in i.c.-inoculated mice, a significant PFC response was discernible from the 6th day, while in i.p. route it was from the first day.

DV replicates in Mϕ, induces immunosuppression to homologous and heterologous antigens with absence of CMI, causes splenic atrophy, depression of phagocytosis, migration and attachment by Mϕ (Tandon *et al.*, 1979; Chaturvedi *et al*, 1978a; Chaturvedi, 1986). The present study shows that DV replicates both in I-A-positive and I-A-negative Mϕ. Removal of the I-A-positive Mϕ had no significant affect on the replication of DV in Mϕ; the reduction in the proportion of antigen-positive cells

being similar to the proportion of the I-A-positive cells. Further, treatment with anti-I-A antibody did not inhibit the uptake and replication of DV in Mϕ. Thus Ia antigens do not appear to be a receptor for DV. On the other hand, the receptors for Semliki virus and LDV on mouse Mϕ are the Ia antigen (Helenius *et al.*, 1978; Oldstone *et al.*, 1980; Inada & Mims, 1984, 1985). Acute LDV infection in mice impairs the capacity of Mϕ to present antigen for reactivation of memory T cells, but without any effect on their phagocytic functions (Isakov, Feldmann & Segal, 1982a b; Inada & Mims, 1984). Another point which merits discussion is the role of I-A-positive Mϕ in the antigen presentation function in the present model. It was observed that depletion of I-A-positive Mϕ did not materially affect the presentation of DV antigen to B cells. In contrast, treatment with CF, which kills nearly 70% Mϕ, none being I-A positive (Chaturvedi *et al.*, 1981a, b, 1987a), depressed antigen presentation by 65% at 3 hr, as the cells which could replicate the virus and present it to B cells were reduced in number. This further supports the absence of any importance of I-A-positive Mϕ in presentation of DV antigen. It is widely accepted that the T cells recognize antigen in the context of cell-surface Ia antigen, while B cells do not have such requirements and recognize antigen alone (Hodes & Singer, 1984; Unanue *et al.*, 1984; Chaturvedi, Rizvi & Mathur, 1987b). This is confirmed by the present model, as both the I-A-negative and -positive Mϕ replicate DV, express its antigen on their surface and present it to B cells, leading to their clonal expansion.

It may be expected that selective infection of antigen-presenting Mϕ which damages these cells may perturb the immune response to the infecting virus and other antigens. Therefore, the capacity of DV-infected Mϕ to present an unrelated antigen was investigated in a Mackaness type of experiment. For this purpose CoxBV was chosen, as it replicates in mouse Mϕ and generates a good PFC response in spleen cells (Tandon *et al.*, 1978). By superimposing CoxBV on DV-infected Mϕ, the interesting phenomenon observed was the impairment of the capacity of the Mϕ to present both the homologous (DV) and heterologous (CoxBV) antigens to B cells. This effect was much more marked at early periods, being up to 68% for DV and 50% for CoxBV responses. Could this be due to antigenic competition? But it was observed that CoxBV infection had no effect on the proportion of Mϕ positive for DV antigen (Fig. 6). The mechanism of the mutual interference by the two viruses in antigenic presentation is not clear and is being investigated. Thus, the capacity of Mϕ to present homologous antigen remained intact but the impairment in antigen presentation was manifested when the Mϕ were exposed to another antigen. Therefore, a particular virus which replicates in Mϕ may not impair its capacity to present its own antigen, especially to B cells. At the time antigen presentation to T cells may seriously be affected as it may require processing of the virus antigen by various pathways (Mills, 1986). Such impairment of antigen presentation to T cells can be a factor in reduced or absent CMI in some of the viral infections, including that by DV.

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