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Variable effects of dengue virus-induced cytotoxic factors on different subpopulations of macrophages

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SUMMARY

Dengue virus (DV) induces T lymphocytes of the spleen to produce a cytotoxic factor (CF) that induces a subpopulation of macrophages (Mϕ) to produce a soluble cytotoxin (CF_2). Both these factors kill normal lymphoid cells and Mϕ. The present study was undertaken to investigate the effects of these factors on the I-A-positive and I-A-negative subpopulations of mouse peritoneal Mϕ. It was observed that CF kills I-A-negative Mϕ and induces I-A-positive Mϕ to produce the CF_2 that kills both types of cells. However, even when combined together, CF and CF_2 do not kill 100% of the Mϕ. The two-step mechanism involving co-operation between T cells and Mϕ appears to be biologically economical for maintaining the cytotoxic pathway.

INTRODUCTION

Macrophages (Mϕ) are specialized phagocytic cells that are widely distributed in the body tissues and play a central role in immune response and body defence. At the same time a number of viruses, including dengue, preferentially replicate in Mϕ (reviewed by Chaturvedi, Nagar & Mathur, 1983b; Morahan, Connor & Leary, 1985; Sissons & Borysiewicz, 1985). Mϕ secrete a large variety of active substances into their milieu that play a biologically important role but, due to developmental or environmental control, they do not secrete all the products simultaneously. A number of these secretory products can lyse target cells, namely arginase, C3a, superoxide, hydrogen peroxide (reviewed by Werb *et al.*, 1986), tumour necrosis factor (Ruff & Gifford, 1981) and cytotoxic factors (Aksamit & Kim, 1979; Takeda *et al.*, 1985).

Dengue type-2 virus (DV) infection in mice induces a cytotoxic pathway in spleen that results in a state of non-specific immunosuppression. The cytotoxic pathway involves induction of a subpopulation of T lymphocytes by DV to produce a cytotoxic factor (CF) in the spleen (Chaturvedi, Bhargava & Mathur, 1980a; Chaturvedi, Dalakoti & Mathur, 1980b). CF kills normal lymphoid cells *in vitro* and *in vivo*; the cells killed are about two-thirds of the Mϕ and one-third of the T cells (Chaturvedi *et al.*, 1981a, b). The Mϕ that survive the killing effect of CF are induced to produce another cytotoxin (CF_2) *in vitro* and *in vivo*, having a cytotoxic effect similar to that of CF (Gulati, Chaturvedi & Mathur 1983a, b, c, 1986). The DV-induced cytotoxic pathway and its effects have been summarized elsewhere (Chaturvedi, 1986).

Different subpopulations of Mϕ have been identified on the basis of their Ia antigens (Cowing, Schwartz & Dickler, 1978) or Mac antigens (reviewed by Unkeless & Springer, 1986). We have observed that blood monocytes and one-third of peritoneal Mϕ are not killed by CF (Chaturvedi *et al.*, 1981a; Chaturvedi, Gulati & Mathur, 1982a). Therefore, it was considered worthwhile to investigate which subpopulation of Mϕ, in terms of Ia antigen, is killed by CF and CF_2, and which produces CF_2.

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MATERIALS AND METHODS

Animals

Adult inbred Swiss albino mice aged 2–4 months were used in the study.

Virus

Dengue type-2 virus (DV), strain P23085, was used in the form of infected mouse brain suspension (Chaturvedi, Tandon & Mathur, 1977).

Preparation of Mϕ monolayers

The peritoneal cavity of normal mouse was lavaged with 5 ml heparinized MEM, and after washing the cells were layered in petri-dishes or on 20×20 mm glass coverslips placed in petri-dishes. After incubation for 2 hr at 37° in an atmosphere of 5% CO_2, the petri-dishes were gently flushed with MEM to remove glass non-adherent cells (Chaturvedi, Shukla & Mathur, 1982b). Among the glass-adherent cells, $96 \pm 2\%$ had phagocytosed

latex particles indicating the proportion of Mϕ. These Mϕ monolayers were used in the experiments.

Preparation of CF

The CF was prepared from the spleen homogenate of DV-infected mice as described elsewhere (Chaturvedi *et al.*, 1980a; Gulati *et al.*, 1983a). The 50% toxicity titre of CF was 2560. In all experiments saturating levels of CF (1:30 dilution) were used.

Preparation of CF_2

The CF_2 was prepared from the normal mouse peritoneal Mϕ monolayers stimulated with CF as described earlier (Gulati *et al.*, 1983a, b). Briefly, 1 ml CF (1:30) was layered on a Mϕ monolayer in a petri-dish and incubated for 1 hr at 4 . The cell sheet was thoroughly washed with MEM and the cells were scraped off with the help of a policeman rod and homogenized in 2 ml PBS, pH 7·0. The clear homogenate obtained after centrifugation at 2000 *g* for 10 min was stored at −20 in small aliquots and used as CF_2. The 50% toxicity titre of CF_2 was 32. In all experiments a saturating level (1:4 dilution) of CF_2 was used.

Assay of cytotoxic activity

The cytotoxic activity of CF and CF_2 was assayed using a single cell suspension of normal mouse spleen as target (Chaturvedi *et al.*, 1980a, b). Briefly, 0·1 ml of the test solution was added to 2×10^6 spleen cells suspended in 0·1 ml MEM. It was incubated at 4 for 1 hr, and non-viable nucleated cells were counted (as a percentage) using trypan blue dye.

Treatment of Mϕ monolayers with antisera and complement

The Mϕ monolayers were treated with anti-I-A^k, anti-I-E^k, or anti-I-A^d antibody (the monoclonal antibodies were kindly supplied by Dr Marc Feldmann, London) and complement (Tandon, Chaturvedi & Mathur, 1979). The optimum dilution of the antibody was established in preliminary experiments. It was observed that treatment with anti-I-A^k antibody and complement killed Mϕ, therefore only this antibody was used in further experiments and has been presented as I-A positive.

All the experiments were set in triplicate and were repeated. Mean values ± SD of the findings from different experiments have been presented and the data have been analysed using Student's *t*-test for *P* values.

RESULTS

CF kills I-A-negative cells

This experiment was done to investigate whether the I-A-positive or I-A-negative Mϕ are selectively killed by the CF. The Mϕ monolayers pretreated with anti-I-A^k antibody and complement were treated with 0·2 ml of CF (1:30 dilution) at 4 for 1 hr and the non-viable cells were counted. The findings presented in Fig. 1 show that treatment with anti-I-A^k antibody + C killed 13 ± 2% of cells and CF killed 57 ± 5%. The proportion of non-viable cells was increased to 69 ± 4% when cells pretreated with anti-I-A^k antibody and complement were treated with CF, the extent of increased cell killing being similar to that by anti-I-A^k antibody + C treatment. Similar results of increased cell killing were obtained when cells pretreated with CF were exposed to anti-I-A^k antibody + C. This shows that I-A-positive cells are not killed by CF.

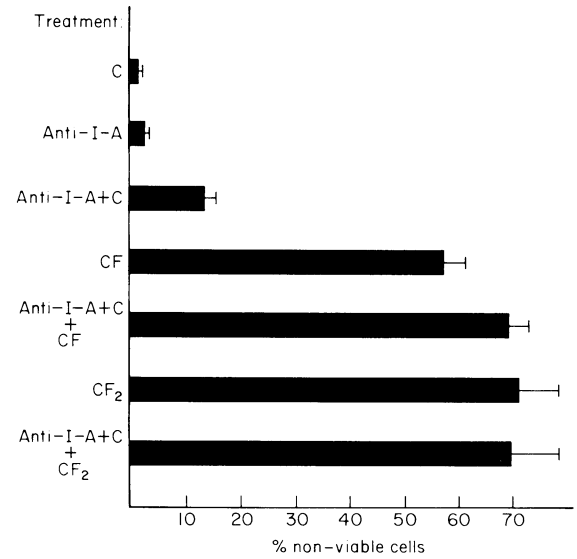

Figure 1. Cytotoxic activity of CF and CF_2 on Mϕ monolayers pretreated with anti-I-A^k antibody (anti-I-A) and complement (C) to deplete I-A-positive cells, or on untreated cells.

CF_2 is produced by I-A-positive cells

An effort was made to study whether CF_2 is produced by I-A-positive or -negative Mϕ. The Mϕ monolayers were pretreated with anti-I-A^k antibody and complement to deplete I-A-positive Mϕ. The cell sheet was washed and incubated with 1 ml CF to induce the production of CF_2 as described in the Materials and Methods. The product was assayed for cytotoxic activity and the findings were compared with those from untreated Mϕ monolayers. The data presented in Fig. 2 show that the CF fails to induce the production of CF_2 in the absence of I-A-positive cells.

CF_2 kills I-A-positive and -negative cells

The Mϕ monolayers were treated with anti-I-A^k antibody and complement followed by treatment with 0·2 ml of CF_2 at 4 for 1 hr. Then the proportion of cells made non-viable was ascertained using trypan blue dye. Mϕ monolayers pretreated similarly with PBS in place of antibody and complement were treated with CF_2. The data presented in Fig. 1 show that the proportion of cells killed in the two groups was similar, and that no additional cells were killed by pretreatment with the antibody and complement. In another experiment 79 ± 8% of cells were killed when the Mϕ monolayer was treated with CF and CF_2 simultaneously.

Immunofluorescent study for I-A-positive cells

Mϕ monolayers pretreated with CF or CF_2, as in the above experiments, were studied for the presence of I-A-positive cells by the indirect immunofluorescent technique (Gardner & McQuillin, 1981; Chaturvedi *et al.*, 1983b). The data presented

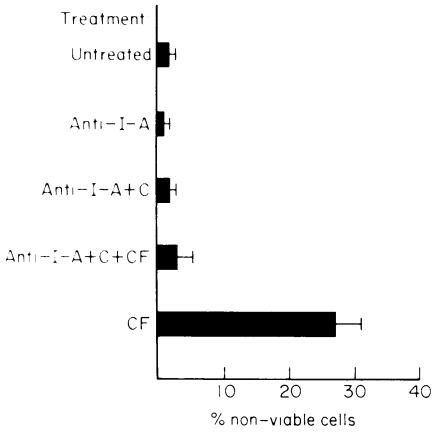

Figure 2. Production of CF_2 by Mϕ on induction by CF. The homogenate of untreated, anti-I-A^k antibody with or without complement (C)-treated Mϕ was not cytotoxic as assayed on normal mouse spleen cell suspension. Mϕ monolayer depleted of I-A-positive cells induced by CF (anti-I-A+C+CF) did not produce CF_2, while untreated Mϕ did produce it.

Table 1. I-A antigen-positive immunofluorescence cells in Mϕ monolayers treated with CF or CF_2

Treatment of Mϕ monolayer	% positive
CF	10±4
CF_2	1±0·5
Untreated	12±5

in Table 1 show the depletion of I-A-positive cells in CF_2-treated Mϕ monolayers while CF had no such effect.

CF_2 is adsorbed by I-A-positive and -negative cells

We have observed that CF_2 gets adsorbed (Gulati *et al.*, 1983b) on the target Mϕ but CF does not (Chaturvedi *et al.*, 1981a). The present experiment was therefore done to examine whether the adsorption of CF_2 is selectively on one type of cells or on both. CF_2 was layered on Mϕ monolayers, which were untreated or pretreated with anti-I-A^k antibody and complement. After incubation at 4 for 1 hr, CF_2 was decanted, spun and assayed for cytotoxic activity. The findings presented in Fig. 3 show that the cytotoxic activity of CF_2 is adsorbed out by both the treated and untreated Mϕ.

Anti-I-A^k antibody does not neutralize cytotoxicity

The effects of anti-I-A^k antibody plus complement treatment, as observed in the previous experiments, could be due to neutralization of the cytotoxicity due to a direct effect on CF or CF_2. In order to study this, the CF or CF_2 was mixed with equal volumes of anti-I-A^k antibody and complement, and incubated at 4 for 1 hr and then at 37 for 30 min. The cytotoxicities of the treated

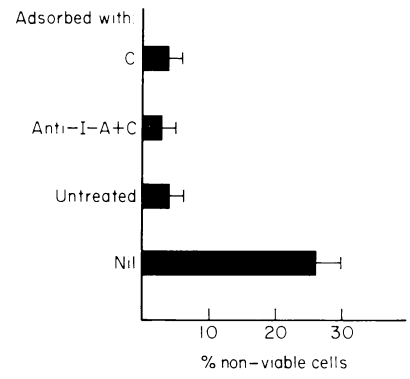

Figure 3. Cytotoxic activity of CF_2 assayed on normal mouse spleen cell suspension after adsorption on Mϕ monolayers that were untreated or pretreated with complement (C) or anti-I-A^k antibody+C or without any adsorption (Nil).

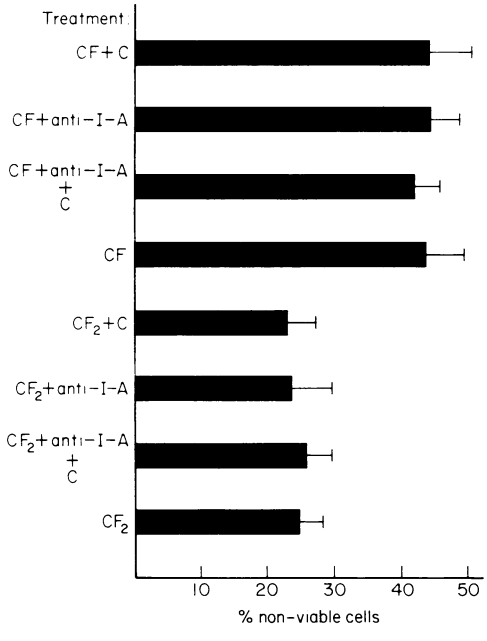

Figure 4. Cytotoxic activity of CF and CF_2, assayed on normal mouse spleen cell suspension, following treatment with C or I-A or I-A+C.

and untreated samples were assayed. The findings presented in Fig. 4 show that the antibody did not neutralize the activity of CF or CF_2.

DISCUSSION

At least two subpopulations of Mϕ have been identified: one expresses the Ia molecule at the cell surface while the other does not. The interesting point generated in the present study is that the two subpopulations of Mϕ are affected differently in the DV-induced cytotoxic pathway: CF kills I-A-negative Mϕ and induces I-A-positive Mϕ to produce CF_2, which kills both types of cells.

The proportion of Ia-positive Mϕ in resident peritoneal Mϕ population has been shown to be 1–5% by Ziegler, Staffileno & Wentworth (1984) and less than 15% by Niederhuber (1978). In

our studies 13 ± 2% resident peritoneal Mϕ were I-A-positive by cytotoxicity assay and 12 ± 5% by immunofluorescent technique. CF kills all the susceptible target cells in the first contact, and re-exposure of cells to additional CF does not enhance the killing (Chaturvedi *et al.*, 1980a, 1981a). However, in anti-I-A^k antibody + C-treated monolayers the CF kills additional cells (I-A-negative cells). Similarly, when CF-treated cells were further exposed to anti-I-A^k antibody + C, an additional percentage of cells were killed, the latter being similar to that by treatment with anti-I-A^k antibody + C alone. The findings of the immunofluorescence study for the demonstration of I-A-positive cells confirmed these findings (Table 1). This shows that CF spares all the I-A-positive and some of the I-A-negative Mϕ.

The Mϕ that survive the killing effect of CF are induced to produce CF (Gulati *et al.*, 1983a, b). The findings of the present study demonstrate that, in the absence of I-A-positive Mϕ, CF fails to induce production of CF_2. Thus, I-A-positive Mϕ are essential for CF_2 production. With the available data one cannot say with certainty whether the I-A-positive Mϕ themselves produce CF_2, or whether they simply help other Mϕ to produce it. In order to resolve this question it is necessary to perform experiments with an I-A-positive subpopulation of Mϕ. Thus, in the DV model, the I-A-positive Mϕ are essential for the transmission of signals of the suppressor pathway (Shukla & Chaturvedi, 1986) and for the production of CF_2, as shown in the present study.

The targets of CF_2 are I-A-positive as well as -negative Mϕ, which adsorb CF_2 and get killed. These findings raise another question as to why CF kills one subpopulation of Mϕ and CF_2 kills both. Interactions between cells that require direct contact are likely to be slow, and only a limited number of cells can be affected. The production of soluble factors is more efficient (Asherson & Zembala, 1976). It has been suggested that the product of one T cell is capable of inducing up to 100 Mϕ cells (Waksman, 1979), therefore a two-step mechanism involving two soluble factors is economical in the amplification of the effect. The selectivity required to maintain this has been shown in the present study.

We have often asked ourselves why Mϕ should produce a substance that results in their own destruction (Gulati *et al.*, 1983a). While trying to answer this question, we have observed that surviving Mϕ become activated by CF in 45 min and continue to produce CF_2 for up to 24 hr without the need of any further CF. Then the Mϕ become refractory to the action of CF for 3 days. By the fourth day Mϕ regain the capacity to produce CF_2 upon the re-induction by CF (Chaturvedi, Gulati & Mathur, 1983a). Thus, it appears that the Mϕ that produce CF_2 become resistant to autodestruction. Further, the findings of the present study show that, out of the glass-adherent population of peritoneal cells, which comprised 96 ± 2% Mϕ, about 40% of the cells survive the killing effect of CF, and about 28% the killing effect of CF_2. On the other hand, the two combined together spare about 17% of the cells.

Mϕ are the principal cells to replicate DV (reviewed by Chaturvedi *et al.*, 1983b), therefore we believe that the body makes an effort to eliminate virus-permissive cells as part of the defence mechanism. This is done by induction of the cytotoxic pathway, which is capable of eliminating Mϕ but this is not 100% effective. In DV-infected mice fewer than 1% of the peritoneal Mϕ show the presence of the virus antigen by the fluorescent antibody technique (Chaturvedi *et al.*, 1983b), while up to 50% of the Mϕ could be infected *in vitro* (unpublished data), therefore there is no need to kill all the macrophages. Blood monocytes are not killed by CF or CF_2 (Chaturvedi et al., 1981a, 1982a; Gulati, Chaturvedi & Mathur, 1984a). These factors act at the plasma membrane of the cell as their effect can be inhibited by treatment of the cells with plasma membrane stabilizing drugs (Gulati *et al.*, 1983c). The putative receptor for CF appears to be a glycoprotein and that of CF_2 sialic acid groups (Gulati, Chaturvedi & Mathur, 1984b). The factors that make some of the Mϕ monocytes resistant to the effect of DV-induced cytotoxic factors remain to be investigated.

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