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Dengue virus-induced cytotoxic factor suppresses immune response of mice to sheep erythrocytes

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Summary. The present study was undertaken to investigate if the suppressed cell-mediated immune responses observed in dengue type 2 virus (DV)-infected mice could be due to the cytotoxic factor (CF) produced in the spleens of DV-infected mice. We have observed that CF given intravenously (i.v.) kills splenic cells and reduces the total cells in the spleen. Mice treated with CF have a significantly depressed immune response to sheep erythrocytes, viz. delayed-type-hypersensitivity as measured by footpad swelling reaction at 24 hr; Jerne's antibody plaque-forming cells in the spleen; and migration inhibition of spleen cells in presence of antigen. These findings are similar to those seen earlier in DV-infected mice.

INTRODUCTION

We have observed the production of a cytotoxic factor (CF) by the T lymphocytes of the spleen of dengue type 2 virus (DV)-infected mice (Chaturvedi, Bhargava & Mathur, 1980a) which kills a variety of normal mice spleen cells *in vitro*, except B lymphocytes (Chaturvedi, Mathur, Gulati & Mathur, 1981a). CF is a heat labile, non-dialysable, macromolecular protein and is destroyed at acidic and alkaline pH (Chaturvedi, Dalakoti & Mathur, 1980b). In earlier studies we have reported lack of cell-mediated immune response in DV-infected mice (Chaturvedi, Tandon & Mathur, 1977; Chaturvedi, Tandon, Mathur & Kumar, 1978b). Study of the immune response of DV-infected mice revealed poor helper and effector T-cell functions as shown by reduced T lymphocytes in the spleen, reduced haemolysin titre following intraperitoneal (i.p.) immunization with sheep erythrocytes, and reduced GVH reactivity of infected spleen cells; but suppressor T cells were present (Tandon, Chaturvedi & Mathur, 1979a). The period of peak cytotoxic activity coincided with the maximum T-cell depletion in DV-infected mice. The relationship between these two phenomena was not understood. The present study was, therefore, undertaken to investigate whether the administration of CF to mice reproduced some of the effects of DV-infection on T-cell functions. Our findings show that CF suppresses the immune response of mice to sheep erythrocytes (SRBC), a thymus-dependent antigen.

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MATERIALS AND METHODS

Animals

The studies were carried out on Swiss albino mice aged

4–6 months and were obtained from the mouse colony maintained in this Department.

Preparation of the cytotoxic factor (CF)
The preparation of CF has been described earlier (Chaturvedi *et al.*, 1980 a,b). The details of the virus and the experimental model have been described (Chaturvedi *et al.*, 1977, 1978b).

Study of the effect of CF on spleen cells in vivo
Mice were inoculated intravenously (i.v.) with 0·25 ml of CF. Thirty minutes and 1, 2, 3, 4 and 24 hr later groups of mice were killed and the spleens were removed. The spleen cells were teased out with forceps in chilled MEM supplemented with 10% foetal calf serum (Chaturvedi, Tandon & Mathur, 1978a). Viable nucleated cells were counted using trypan blue dye exclusion. The percentage of non-viable cells was calculated after counting 300–400 spleen cells. The cells were kept on ice throughout. Control mice received 0·25 ml of normal mouse spleen homogenates. The non-viable cells have been expressed as the mean value of duplicate tests on 5–6 mice after deducting the background of non-viable cells obtained in the control mice.

Antigen
Sheep erythrocytes (SRBC) in Alsever's solution were obtained from the animals maintained in this Department and washed thrice in phosphate-buffered saline (PBS) before use. For priming, the mice were injected intraperitoneally with 4×10^8 SRBC suspended in 0·2 ml PBS.

Experimental design
The immune response of CF-treated and matched control mice to SRBC was investigated. Mice were given CF in doses of 0·25 ml i.v. followed 24 hr later by 4×10^8 SRBC i.p. Further, mice were given 0·25 ml CF i.p. at intervals of 48 hr until the termination of experiments at 4, 7 and 15 days after the immunization with SRBC. The control mice were similarly treated with normal spleen homogenate. The dose effect of CF was studied at those time periods when the immune response was maximal.

Delayed-type hypersensitivity
The delayed-type hypersensitivity (DTH) response was determined as footpad swelling reaction (FPR) of mice primed with SRBC. At different periods after priming, as described in the results, mice were challenged with $2{\cdot}5 \times 10^8$ SRBC suspended in 0·025 ml PBS in the footpad of hind paws. Increase in footpad thickness was measured with a dial gauge calliper ('Oditest', M.C. Kroplin GMBH, Messzeugfabrik, West Germany) by measurement before and 24 hr after challenge with SRBC. The extent of non-specific inflammatory response to injection with SRBC challenge dose only was measured in the footpads of non-immunized controls of mice receiving CF and control spleen homogenate.

Leucocyte migration inhibition (LMI) test
This test was performed with mouse spleen cells. Details have been described elsewhere (Chaturvedi *et al.*, 1978b). The percentage migration inhibition was calculated from the area of cell migration with and without SRBC antigen.

Antibody plaque-forming cells (PFC)
The spleens were collected and viable nucleated cells were counted. Direct PFC against SRBC were counted by the haemolytic plaque technique of Jerne & Nordin (1963) as described elsewhere (Chaturvedi *et al.*, 1977; Tandon & Chaturvedi, 1977). Multiple slides were prepared from each mouse spleen.

The data were analysed by the Student's *t* test.

RESULTS

Effect of CF on spleen cells *in vivo*
Findings presented in Table 1 s how a statistically significant decline ($P = < 0{\cdot}001$) in the total number of spleen cells in CF treated mice. A count of the non-viable cells present in the spleen cell suspension revealed that CF killed $45 \pm 1{\cdot}5\%$ cells in 3 hr (Table 1). In another experiment it was observed that a single dose of CF given intraperitoneally to assess the number of T cells affected by CF. Three hours after CF i.v. percentage of T cells in spleen cell suspensions was assayed by counting the number of cells made non-viable by treatment with antithymocyte serum and complement as described earlier (Tandon *et al.*, 1979a). More cells ($26 \pm 3\%$) of CF-treated mice and $38 \pm 6\%$ cells of normal mice spleen were made non-viable by this treatment.

Effect of CF treatment on DTH responses
The DTH response to challenge with SRBC of control and CF treated mice 4, 7 and 15 days after immunization with SRBC, are summarized in Fig. 1. The increase in footpad thickness was significantly suppressed ($P = < 0{\cdot}05$ to $< 0{\cdot}005$) at all times after CF

Table 1. Effect of CF on spleen cells *in vivo**

Period (hr)	Total cells per spleen ($\times 10^8$)	P†	Non-viable cells (%)
CF-treated mice			
30 min	4·1±0·3	<0·001	12±2
1	3·5±0·2	<0·001	19±5
2	2·5±0·1	<0·001	35±6
3	3·3±0·2	<0·001	45±1·5
4	2·6±0·2	<0·001	25±1
24	1·7±0·1	<0·001	23±1
Control mice	5·40±0·2		6±2

* Killing of cells in the spleen of mice given a single dose of CF i.v. At different periods the spleens were taken out and the cells were teased out in chilled MEM. Total number and the percentage non-viable cells were counted in the single-cell suspension using trypan blue dye.

† P value for mice treated with CF compared with control mice.

injection. High dilutions of $10^{-3·7}$ and 10^{-4} CF produced insignificant suppression ($P = >0·05$). Non-specific inflammatory responses to injection of the SRBC challenge dose alone in the footpads of non-immunized controls was negligible (3–5%) and was similar in the two groups.

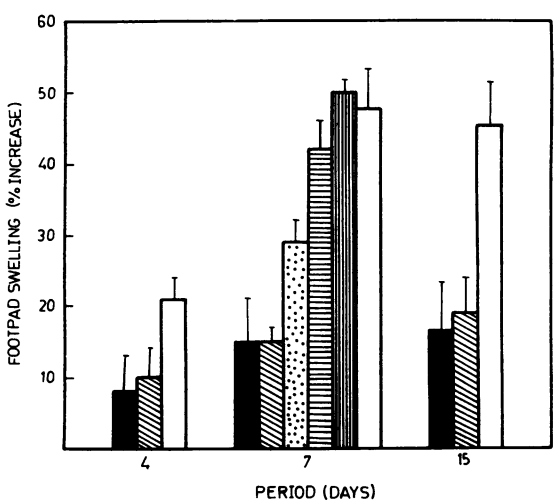

Figure 1. Suppressed delayed type-hypersensitivity in mice treated with CF. SRBC (4×10^8) were injected i.p. in control mice (□), and mice which had received CF i.v. 24 hr earlier and then i.p. at 48-hr intervals. Twenty-four hour footpad swelling reaction to SRBC of mice sensitized with SRBC 4, 7 and 15 days previously was measured. Each column represents mean value with standard error of the mean of five to ten readings. ■, Mice given CF diluted 10^{-1}; ▨ columns, mice given CF diluted 10^{-2}; ▨ columns, mice given CF diluted 10^{-3}; ▤, mice given CF diluted $10^{-3·7}$; ▥, mice given CF diluted 10^{-4}.

Effect of CF treatment on PFC

Another series of experiments were performed to measure the T-cell dependent primary antibody response to SRBC as measured by PFC counts in control and CF-treated mice. The results are presented in Fig. 2. Four days after inoculation the immunoglobulin M PFC responses to SRBC by spleen cells from CF-treated mice were decreased by 20% of the response by control mice spleen cells ($P = <0·05$). The response of mice treated with varying dilutions of CF was studied at day 7. A significant suppression ($P = <0·005$) of PFC was observed with CF diluted up to 10^{-3} (Fig. 3). At day 15 the PFC response was minimal and was similar in treated and control groups.

Effect of CF on leucocyte migration inhibition (LMI)

In the next series of experiments, presented in Fig. 3, the cell-mediated immune response as shown by LMI and by SRBC was compared in CF-treated and control mice. The mean spleen cell migration inhibition in CF-treated mice was significantly less ($P = <0·005$ to $0·001$) than that of controls at all the three periods of observation with 10^{-1} and 10^{-2} dilutions of CF. The dose response was investigated at day 15. A significant suppression of the response ($P = <0·005$ to $<0·001$) was observed with CF diluted up to 10^{-3}. With dilutions greater than $10^{-3·7}$ the effect was insignificant ($P = >0·05$).

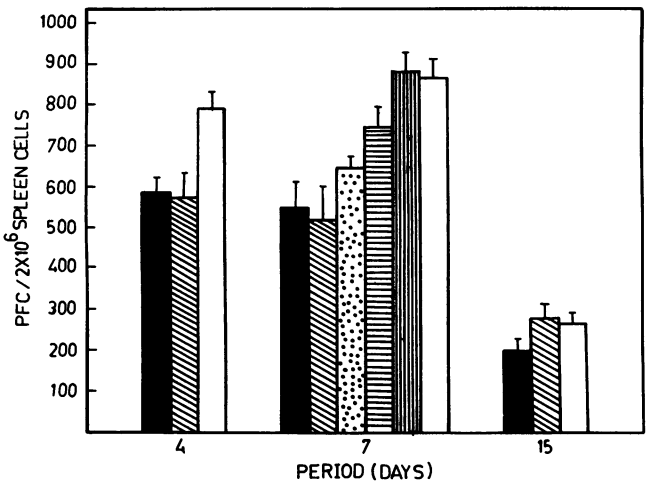

Figure 2. Suppression of antibody plaque forming cells (PFC) in the spleen of mice treated with CF. SRBC (4×10^8) were injected i.p. in control mice (□), and mice which had received CF i.v. 24 hr earlier and then i.p. at 48-hr intervals. Jerne's PFC against SRBC were counted at 4, 7 and 15 days after priming with SRBC. Each column represents mean values with standard error of the mean of fifteen to eighteen observations. ■, Mice given CF diluted 10^{-1}; ▨, mice given CF diluted 10^{-2}; ▩, mice given CF diluted 10^{-3}; ▤, mice given CF diluted $10^{-3\cdot7}$; ▥, mice given CF diluted 10^{-4}.

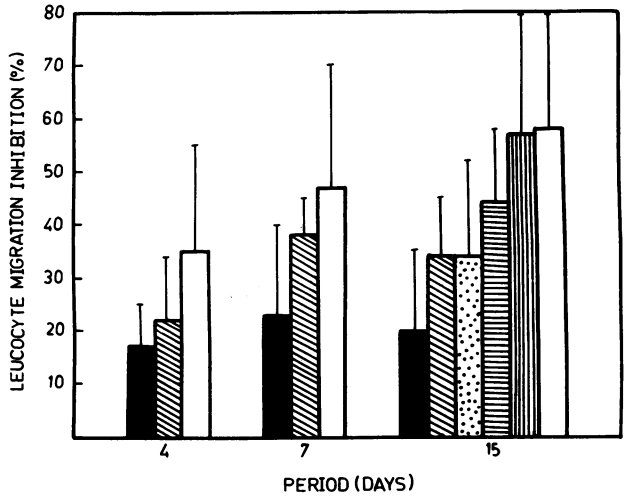

Figure 3. Suppression of migration inhibition of spleen cells obtained from CF-treated mice in presence of SRBC antigen. SRBC (4×10^8) were injected i.p. in control mice (□) and mice which had received CF i.v. 24 hr earlier and then i.p. at 48 hr intervals. Migration inhibition of spleen cells in presence of SRBC antigen was assayed at 4, 7 and 15 days after immunization with SRBC. Each column represents mean value of five to ten observations with standard error of the mean. ■, Mice given CF diluted 10^{-1}; ▨, mice given CF diluted 10^{-2}; ▩, mice given CF diluted 10^{-3}; ▤, mice given CF diluted $10^{-3\cdot7}$; ▥, mice given CF diluted 10^{-4}.

DISCUSSION

The data presented here show that parenteral administration of CF to mice kills spleen cells. Furthermore CF killed about one third of the T lymphocytes of spleen *in vivo*. This is similar to our earlier *in vitro* findings that CF killed about one third of the T lymphocytes and most of the macrophages besides other cells of the spleen. CF is equally effective in killing lymph node cells and kills about 11% of thymic cells (Chaturvedi *et al.*, 1981b). Could some of the reduced immune responses in DV-infected mice be due to killing of cells in the spleen and elsewhere in the body by the CF? We have not yet studied all the types of spleen cells killed *in vivo* by CF but there is a temptation to extrapolate the *in vitro* finding to the *in vivo* situation. If it is true, then killing of T cells and macrophages by the CF may explain depressed expression of DTH; depressed helper function effecting PFC and depressed effector function reducing the LMI. The suppression was statistically significant in all the parameters studied but was more marked in DTH and LMI. CF kills most of the macrophages (Chaturvedi *et al.*, 1981a), therefore, immune functions involving them were most affected. Since B cells are not affected by CF (Chaturvedi *et al.*, 1981a) depression of PFC was not so great and appears to be mediated through the effect on helper T cells.

We have observed that all the functions of T-cell-mediated responses tested in the present study appear simultaneously. This is supported by the finding of Hahn, Kaufmann, Falkenberg, Chahin & Horn (1979) who have shown that the helper and DTH T cells to SRBC appear together in mice. It therefore appears that CF simultaneously affects the cell populations responsible for all the cell-mediated responses investigated in the present study. How CF kills the spleen cells is not known. One of the mechanisms by which immune lymphocytes can specifically affect target cell destruction is by secretion of a non-specific substance (Gatey, Mayer, & Henney, 1976). It is possible that CF may be an effector molecule mediating immunosuppression non-specifically in DV-infected mice.

Suppressor cells are present in the spleen of DV-primed mice (Chaturvedi *et al.*, 1978b; Tandon *et al.*, 1979a). The suppressor activity is mediated through splenic T lymphocytes and also by a soluble factor present in the cell-free homogenate of the spleen of DV-infected mice (Tandon, Chaturvedi & Mathur, 1979b; Chaturvedi, Shukla & Mathur, 1981b). Thus, DV-infected mouse spleen homogenate contain both the CF and the suppressor factor (SF). How much of the depressed immune responses observed in the present study can be attributed to CF or the SF is not yet fully known.

Thus our data show that i.v. administration of CF kills and depletes spleen cells of mice resulting in depressed T lymphocyte responses to SRBC. This is similar to the depressed cell-mediated immune response seen in DV-infected mice in our earlier studies.

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