Immunology 1980 **40** 665

Production of cytotoxic factor in the spleen of dengue virus-infected mice

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Accepted for publication 29 February 1980

Summary. Swiss albino mice inoculated i.c. with dengue type 2 virus (DV) show presence of a factor in the spleen which is cytotoxic to the normal mice spleen cells *in vitro*. The cytotoxic factor is present in the homogenate as well as in the culture supernatants of the spleen cells. Maximum cytotoxic activity is present in the spleen of moribund mice on 10–11 p.i. day. No cytotoxic activity was found in the homogenates of brain, liver, heart and skeletal muscles. Sera from a few mice only had cytotoxic activity and were not complement-dependent. Cytotoxic activity in the homogenate and culture supernatant (TCF) of various spleen cell populations was screened. Cytotoxicity of glass-non-adherent cells was significantly higher. DV-infected spleen cells, depleted of macrophages by carbonyl iron treatment, had higher cytotoxic activity. B-cell-enriched fractions and the glass-adherent cells had negligible cytotoxic activity. Purified lymphocytes and the T-lymphocyte-enriched fractions had maximum cytotoxicity. Thus, a cytotoxic factor is produced by T lymphocytes of the spleen of DV-infected mice which kills normal mouse spleen cells *in vitro*.

INTRODUCTION

Studies on the host defence mechanisms in dengue type 2 virus (DV)-infected mice revealed that adoptively transferred sensitized spleen cells failed to protect normal or immunosuppressed mice against challenge with a small dose of DV and anti-thymocyte serum failed to potentiate the *in vitro* migration of spleen cells from DV-infected or sensitized mice (Chaturvedi, Tandon & Mathur, 1977; Chaturvedi, Tandon, Mathur & Kumar, 1978a). These observations indicate a poor cell-mediated immune response. In a subsequent study, it was shown that there is a selective depletion of T-lymphocyte subpopulations responsible for helper and effector functions sparing suppressor cells in the spleens of DV-infected mice (Tandon, Chaturvedi & Mathur, 1979a). The suppressor activity was attributed to T lymphocytes (Tandon, Chaturvedi & Mathur, 1979b).

Depletion of T lymphocytes has been observed in a number of viral infections (reviewed by Woodruff & Woodruff, 1975). This could be due to transient lymphocytopoenia, depletion of lymphocytes in thymus-dependent areas of lymphoid tissue (Woodruff & Woodruff, 1970); changes in T-lymphocyte traffic, (Woodruff & Woodruff, 1974, 1975); or destruction of T lymphocytes directly by the virus (Snodgrass, Lowry & Hanna, 1972). DV has been shown to replicate in monocytes (Halstead & O'Rouke, 1977) and in B lymphocytes but not in T-lymphocytes (Theofilopoulos, Brandt, Russel & Dixon, 1976).

Sera of patients with variety of viral infections have been shown to have a factor, cytotoxic to normal lymphocytes *in vitro* (Huang, Lattos, Nelson, Reeb & Hong, 1973). While looking for the possible mechanism of T-cell depletion in DV-infected mice, it

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0019-2805/80/0800-0665$02.00

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was observed that sera of a few DV-infected mice had a cytotoxic effect on normal mouse spleen cells *in vitro*. This has lead to detailed investigation of a cytotoxic factor produced in the spleen of DV-infected mice.

MATERIALS AND METHODS

Details of the dengue type 2 virus (DV) and the adult mice used in the study have been described elsewhere (Tandon & Chaturvedi, 1977; Chaturvedi *et al.*, 1977; Agrawal, Tandon, Chaturvedi & Kurmar, 1978). A dose of 1000 LD50 intracerebrally (i.c.) of the virus was used throughout the study.

Preparation of spleen cells

The spleen cells were teased out in cold MEM (Chaturvedi, Tandon & Mathur, 1978b). The single cell suspension was washed and the cells were treated with Tris–ammonium chloride pH 7·2 to remove the erythrocytes. The cells were washed and viable nucleated cells were counted by the trypan blue dye exclusion test. Viability varied from 90 to 95%.

Preparation of enriched cell populations:

Macrophages were removed from the spleen cell suspension by carbonyl iron (Lymphocyte Separator Reagent, Technicon Instruments Corp., N.Y.) treatment (Tandon *et al.*, 1979b). The lymphocyte-rich population was obtained by gradient centrifugation on Lymphoprep (sodium metrizoate and Ficoll, density 1·077 g/ml, Nyegaard & Co., AS., Oslo) of macrophage-depleted spleen cells (Chaturvedi, Mathur, Tandon, Natu, Rajvanshi & Tandon, 1979). T- and B-lymphocyte-enriched populations were obtained through nylon-wool column (Julius, Simpson & Herzenberg, 1973; Trizio & Cudkowicz, 1974) as described earlier (Tandon *et al.*, 1979a, b; Chaturvedi, Mathur & Mathur, unpublished results). Glass-adherent and glass-non-adherent cells were separated by spreading spleen cell suspensions in petri dish and incubating it at 37° in an atmosphere of CO_2. The two types of cell populations were separated as described elsewhere (Chaturvedi *et al.*, 1978b).

Preparation of spleen homogenate

On different days after the virus inoculation mice were killed and the spleens were collected aseptically, and homogenized to make a 10% suspension in phosphate-buffered saline (PBS) pH 7·2 by an MSE tissue homogenizer with steel blades and glass cups (Tandon, Chaturvedi & Mathur, 1979a). The homogenate was stored at $-20°$ in small aliquots. Normal spleen homogenates were prepared similarly. In some experiments homogenates were prepared from spleen cell suspensions.

Preparation of culture supernatants of spleen cells

2×10^6 viable spleen cells/ml in MEM containing 1% HEPES and 10% foetal calf serum (Armour Pharmaceutical Co., England) were cultured in Leighton tubes and incubated at 37°. After 24 h the preparations were centrifuged at 4000 r.p.m. at 4° and the supernatant tissue culture fluid (TCF) was collected. For control, TCF from similarly cultured normal mouse spleen cells was obtained. In preliminary experiments, it was observed that the cytotoxicity of TCF obtained after 24, 48 or 72 h culture was similar. Therefore in all experiments the cells were cultured for 24 h.

Cytotoxicity test

The cytotoxic activity of the homogenate and the tissue culture supernatant (TCF) was tested using normal mouse spleen cells as target. The tests were carried out in macro-HI perspex trays with 80 round-bottomed wells, using 0·1 ml volume of each preparation. Serial doubling dilutions of the homogenate were prepared in MEM containing 5% foetal calf serum. The target single celled normal mouse spleen cell suspension was prepared as described before. To each dilution of the homogenate 2×10^6 normal mouse spleen cells suspended in 0·1 ml volume were added. After thorough mixing, the trays were kept at 4° in a refrigerator for 1 h. The trays were rocked every 10 min during the period of incubation. Then the non-viable cells were counted using trypan blue dye exclusion test (Chaturvedi *et al.*, 1978b). The cells were evenly suspended and 200–300 cells were counted under high magnification ($\times 400$) of the microscope and the percentage non-viable cells was calculated. With every test a cell control was set up using the diluent in place of the homogenate. Throughout the test all the procedures were carried out at 4° in a ice bath. Every test was run in duplicate or triplicate and the mean value of percentage of non-viable cells $\pm$ standard error of the mean as obtained in repeated experiments (14–20 observations) are presented. The background non-viable cells were deducted while presenting the results. The data have been analysed by Student's t test for P value.

RESULTS

Data presented in Fig. 1 show that the cytotoxic activity of the DV-infected spleen homogenate was negligible in the initial period after virus inoculation. It gradually increased, more so from the 6th day onwards reaching a peak on day 11 of $41 \pm 6\%$. Maximum cytotoxicity of the normal mouse spleen homogenate was $6 \pm 2\%$. The cytotoxic activity of the sera of DV-infected mice has been presented in Fig. 2. In the majority of the mice the cytotoxic activity of the sera was negligible. Cytotoxicity of the normal mice sera was $8 \pm 2{\cdot}5\%$.

Since peak cytotoxicity was found on days 10 and 11 p.i. all other experiments were carried out on spleens collected at this period.

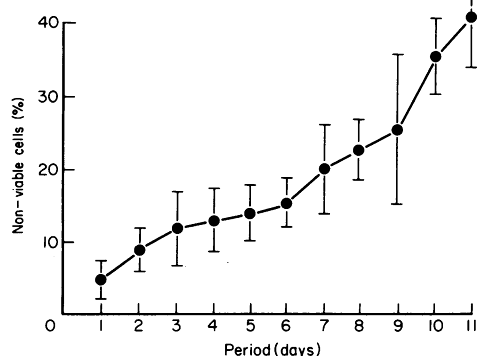

Figure 1. Cytotoxic effect of spleen homogenates from DV-infected mice, obtained at different periods after virus infection. Each point represents mean value ±SEM of 10–14 observations in repeated experiments.

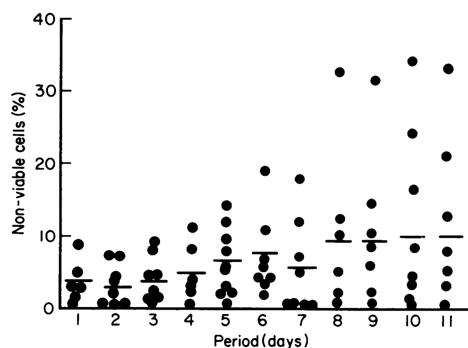

Figure 2. Cytotoxic effect of the sera of DV-infected mice obtained at different periods after virus infection. Each point represents mean value of replicate tests on serum from one mouse. Horizontal line represent mean value at each time interval.

Effect of temperature of incubation on cytotoxicity

The replicate tests were incubated for 1 h at 4° or 37° to see the effect of temperature of incubation on cytotoxicity. Figure 3 shows the cytotoxicity produced by different dilutions of spleen cell homogenates. The findings were similar at the two tempertures.

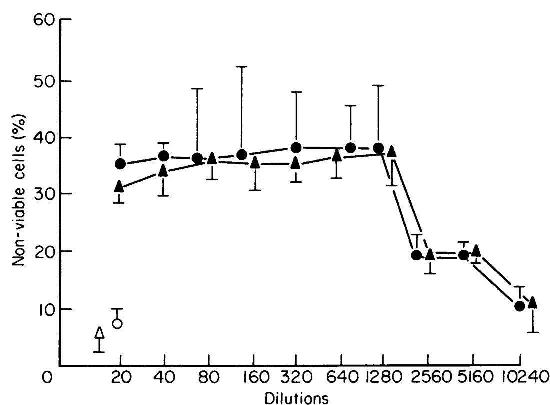

Figure 3. Effect of incubation temperature on cytotoxic activity of DV-infected mouse spleen homogenates. Closed circles, test at 4° with DV-infected homogenate; open circle, test at 4° with normal spleen homogenate; closed triangles, test at 37° with DV-infected homogenate; open triangle, test at 37° with normal spleen homogenate. Each point represents mean value ±SEM of four tests, each in duplicate or triplicate.

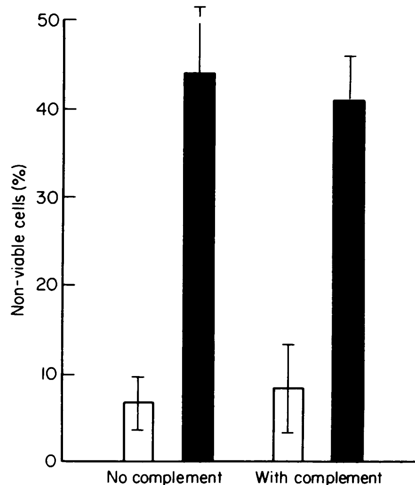

Figure 4. Effect of complement on cytotoxic activity of DV-infected mouse spleen homogenates. Each observation represents mean value ±SEM of 14–18 tests. Open column, normal mice spleen homogenates; filled column, DV-infected mice spleen homogenates.

Effect of complement on cytotoxicity

DV-infected spleen homogenate was incubated with the target cells at 4° for 1 h. To one set of experimients guinea-pig complement (1:6 diluted) was added and to the other set diluent was added in place of complement. After further incubation for 1 h at 37° the non-viable cells were counted. Normal spleen homogenate was included for control. The findings presented in Fig. 4 show that the cytotoxicity in presence of complement was 41 ± 4·5% while it was 44 ± 8% in the set of tests without complement.

Search for cytotoxic factor in other organs

Brain, liver, heart, spleen and skeletal muscles from thigh were collected from severely ill mice and a 10% homogenate was prepared as described above. The doubling dilutions of the homogenates were screened for cytotoxicity. Findings presented in Fig. 5 show that the cytotoxicity of the homogenates of different organs varied from 4 to 8% while that of spleen homogenate was 41 ± 6%.

Cytotoxicity of spleen cell culture supernatant

Spleen cell suspensions obtained from severely sick mice, 11 days after DV infection were divided into two aliquots. One was homogenized and the other was cultured for 24 h. The cytotoxicity of the homogenate and the TCF was compared. Similarly treated cells from normal healthy mice were included for controls. The findings summarized in Fig. 6 show that the cytotoxicity of the DV-infected spleen cell homogenate was 29 ± 5% while that of whole spleen homogenate was 41 ± 6%. The cytotoxic activity of the normal spleen cell homogenate was 4 ± 2%. Similar results were obtained with TCF.

Cytotoxicity of macrophage-depleted spleen cell preparations

The data presented in Fig. 7 show that the cytotoxicity of the homogenate of DV-infected macrophage-depleted cells was 38 ± 8% while that of TCF was

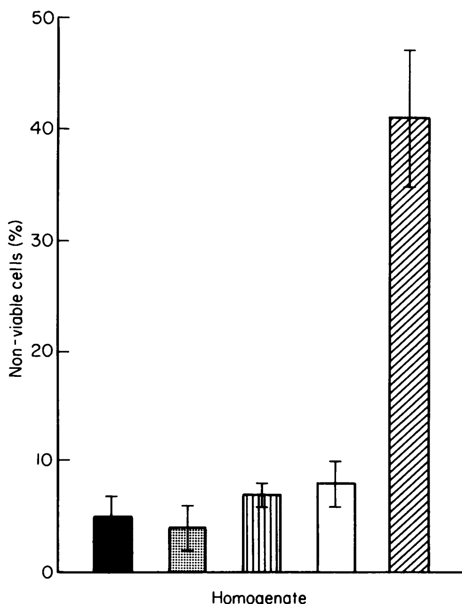

Figure 5. Cytotoxic effect of homogenates from different organs of moribund DV-infected mice. Each observation represents mean value ± SEM of 14–20 tests. Filled column, brain homogenate; stippled column, liver homogenate; vertical striped column, heart homogenate; open column, skeletal muscle homogenate; hatched column, spleen homogenate.

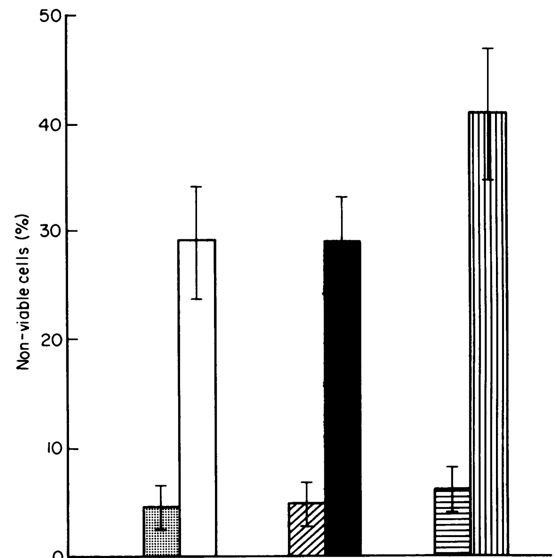

Figure 6. Cytotoxic effect of homogenates of whole spleen, and spleen cell suspensions and the culture supernatant (TCF) of spleen cells from DV-infected mice. Each observation represents mean value ± SEM of 14–20 tests. Stippled column, normal spleen cell homogenate; open column, DV-infected spleen cell homogenate; hatched column, TCF of normal spleen cells; filled column, TCF of DV-infected spleen cells; horizontal striped column, homogenate of whole normal spleen; vertical striped column, homogenate of whole DV-infected spleen.

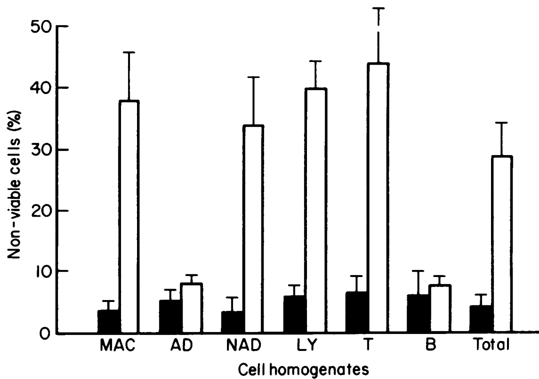

Figure 7. Cytotoxic effect of the homogenates of enriched spleen cell populations from DV-infected mice. Each column represents mean value of 14–18 observations in repeated experiments. Open columns, cells from DV-infected mice; filled columns, cells from normal mice. MAC, macrophage-depleted cells; AD, glass-adherent cells; NAD, glass-non-adherent cells; LY, lymphocyte-enriched cells; T, T-lymphocyte-enriched cells; B, B-lymphocyte-enriched cells; Total, total spleen cells.

34±6% (Fig. 8). The cytotoxicity of the normal macrophage-depleted cell preparations were 3–4%.

Cytotoxicity of glass-adherent cell preparations

Findings presented in Figs 7 and 8 show that cytotoxicity of the DV-infected cell homogenate was 8·2±1·5% and that of its TCF was 7±1·3%. The cytotoxicity of the control cells were 5·3±2%.

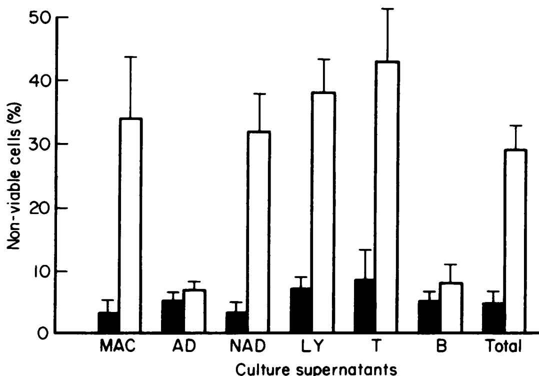

Figure 8. Cytotoxic effect of the culture supernatant of enriched spleen cell populations from DV-infected mice. Each column represents mean value of 14–18 observations in repeated experiments. Open columns, cells from DV-infected mice, filled columns, cells from normal mice. MAC, macrophage-depleted cells; AD, glass-adherent cells; NAD, glass-non-adherent cells; Ly, lymphocyte-enriched cells; T, T-lymphocyte-enriched cells; B, B-lymphocyte-enriched cells; Total, total spleen cells.

Cytotoxicity of glass-non-adherent cell preparations

The cytotoxicity of the homogenate of DV-infected glass-non-adherent cells was 34±8% (Fig. 7) while that of their TCF was 32±6% (Fig. 8).

Cytotoxicity of lymphocytes of spleen

Figure 7 shows that cytotoxicity of the homogenate of DV-infected lymphocyte-enriched preparations was 40±4% while that of total spleen cells was 29±6%. Similar findings were observed with TCF (Fig. 8). Cytotoxicity of normal mouse lymphocytes was 4–6%.

Cytotoxicity of T lymphocyte preparations

The homogenates of T lymphocytes from the spleen of DV-infected mice killed 44±10% spleen cells (Fig. 7) while TCF from the same source killed 43±8% cells (Fig. 8).

Cytotoxicity of B lymphocyte preparations

The cytotoxicity of B lymphocyte homogenates from DV-infected mice spleens was 8±0·6% which was similar to that of homogenates of B cells from normal healthy mice (Fig. 7). Similar findings were obtained with TCF of DV-infected or normal B lymphocytes (Fig. 8).

DISCUSSION

These findings show that a factor cytotoxic to normal mouse spleen cells *in vitro* is produced in the spleens of DV-infected mice. DV replicates in the brain, liver, spleen (Chaturvedi *et al.*, 1977; 1978a), skeletal muscles (Agrawal *et al.*, 1978) and heart (Chaturvedi, Mathur & Mehrotra, 1974; Chaturvedi *et al.*, 1978a) of mice but the cytotoxic factor is found only in spleen homogenates and not in that of orther organs. The whole spleen homogenate is much more cytotoxic ($P \leq 0{\cdot}001$) than that of spleen cell suspension. It may be due to the presence of a cytotoxic factor in the extracellular compartment of spleen which is lost during the process of teasing out and washing of cells. It is likely that some of the free cytotoxic factor is leaked

into the blood as seen in a few mice. The cytotoxicity of the cell homogenate and the TCF was similar in all the experiments, thus showing similar activity of the secreted and intracellular cytotoxic factor.

The incubation temperature of the test had no effect on the extent of cytotoxicity. Incubation at 37° or 4° for 24 h did not enhance the cytotoxicity (unpublished data), but due to higher background non-viability these tests were not pursued. In certain viral infections, the cytoxic factor has been shown to be an immunoglobulin and its effect was complement-dependent (Huang *et al.*, 1973). In the present study, addition of complement in the test had no effect on cytotoxicity.

When cultured lymphoid cells are stimulated by a mitogen or a specific antigen, cytotoxic activity is detected in the culture fluid 3–5 days later and has been termed lymphotoxin (Ruddle & Waksman, 1968; Granger & Kolb, 1968; Jeffes & Granger, 1975). The cytotoxic factor observed in the present study has many apparent differences from classical lymphotoxin, *viz.* stimulation is achieved *in vivo*, production *in vitro* does not require stimulus by additional antigen, the target is lymphoid cells of spleen, and the action on target cells is effected in 1 h at 4°. The factor is similar to the lymphotoxin in being secreted by lymphoid cells on stimulation by an antigen, and the activity is present both in the cells as detected in the cell homogenate, as well as secreted as shown in TCF.

An effort was made to identify the splenic cell type responsible for production of cytotoxic factor in DV-infected mice. Macrophages are not the source of cytotoxic factor because the cytotoxicity of the macrophage-depleted spleen cell preparations was higher than that by total spleen cells ($P = <0{\cdot}001$); cytotoxicity of the glass-adherent cells was negligible while that by the glass-non-adherent cells was significantly higher ($P = <0{\cdot}001$). The findings in several experiments indicated that the source of cytotoxic factor were lymphocytes, *viz.* higher cytotoxicity of the preparations of (i) macrophage-depleted cells; (ii) lymphocyte-enriched fractions and (iii) glass-non-adherent cells. Among the lymphocytes, cytotoxicity of B cells was negligible while the T-lymphocyte preparations had maximum cytotoxicity. It, therefore, appears that T lymphocytes are responsible for the production of cytotoxic factor in the spleen of DV-infected mice. In another study, we have observed that the cytotoxic factor kills mainly macrophages and lymphocytes of normal mouse spleen *in vitro* (Chaturvedi *et al.*, 1980).

It is interesting to note that the cytotoxic activity in the spleen increased gradually after DV i.c., while the T lymphocytes, as observed earlier (Tandon *et al.*, 1979a), gradually decreased reaching lowest values from the 8th day. Among T-cell functions remaining at this period of DV infection were those showing suppressor activity (Tandon *et al.*, 1979a; 1979b). The data presented here suggests that another subpopulation of T lymphocytes surviving DV infection is that responsible for production of the cytotoxic factor. The period of peak cytotoxic activity coincides with maximum T-cell depletion. Whether some of these effects are caused by the cytotoxic factor is being investigated.

ACKNOWLEDGMENT

We sincerely thank Dr Pushpa Tandon for doing some preliminary experiments on serum cytotoxicity.

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