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Characterization of the cytotoxic factor produced in the spleen of dengue virus-infected mice

U. C. CHATURVEDI, HEMLATA DALAKOTI & ASHA MATHUR *Virus Laboratory, Department of Pathology and Bacteriology, K.G. Medical College, Lucknow, India*

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Summary. Data presented in the study show that the cytotoxic factor (CF) produced in the spleen of (dengue-virus) infected mice can be purified by agarose-gel electrophoresis and Sephadex G-100 gel filtration. CF is non-dialysable, heat-labile, trypsin-sensitive, unstable at acidic and alkaline pH, a macromolecular substance which sediments on ultracentrifugation and is retained by a Millipore filter of 0·45 μm size. Its approximate molecular weight is $1{\cdot}15\ (\pm 0{\cdot}34) \times 10^5$ as determined by gel filtration.

INTRODUCTION

The poor cell-mediated immune response observed in dengue virus type 2 (DV)-infected mice (Chaturvedi, Tandon & Mathur, 1977; Chaturvedi, Tandon, Mathur & Kumar, 1978a) has been shown to be due to the selective depletion of T-lymphocyte subpopulations responsible for helper and effector functions but sparing suppressor T cells (Tandon, Chaturvedi & Mathur, 1979a, b). During a study of the mechanism of T-cell depletion in DV-infected mice there was found a factor cytotoxic to normal mouse spleen cells *in vitro*. This cytotoxic factor (CF) was found in the homogenate as well as in the culture supernatant of the spleen cells obtained from the DV-infected mice but not in the homogenate of other organs of such mice. It was also observed that CF was produced by the T lymphocytes of the spleen (Chaturvedi, Bhargava & Mathur, 1980). It killed macrophages, T lymphocytes and few other cells of mouse spleen but had no effect on B lymphocytes. It was further observed that the mice treated with CF have suppressed immune responses to sheep RBC (Chaturvedi *et al.*, unpublished observations). An effort was made to characterize and study the physicochemical properties of CF. The present paper describes some of the findings.

Correspondence: Dr U. C. Chaturvedi, A-4 Thakurganj Colony, Lucknow-226003, India.

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MATERIALS AND METHODS

Animals

Swiss albino mice aged 4–6 months were obtained from the mouse colony maintained in this department.

Virus

Source of the virus was brain suspension of dengue type 2 virus (DV)-infected adult mice (Agrawal, Tandon, Chaturvedi & Kumar, 1978). DV produced a uniformly fatal illness in adult mice on intracerebral (i.c.) inoculation, killing all the mice in 8–11 days. The details of the DV experimental model have been described earlier (Chaturvedi *et al.*, 1977; Tandon & Chaturvedi, 1977; Chaturvedi *et al.*, 1978a).

Preparation of cytotoxic factor (CF)

The details of the preparation of CF have been described earlier (Chaturvedi *et al.*, 1980). Briefly, forty mice were inoculated i.c. with 1000 LD_{50} of the DV. After 10–11 days, the moribund mice were killed and the spleens were removed aseptically. Spleens were washed free of blood and homogenized in a MSE tissue homogenizer giving a 10% solution (w/v) in phosphate-buffered saline, pH 7·0. The homogenate was centrifuged in the cold at 3000 r.p.m. for 10 min and the clear fluid was stored in small aliquots at −20°. This was used as CF. A similar preparation from the homogenates of normal mouse spleens was used as a control in every experiment.

The cytotoxic factor was purified by prolonged agarose-gel electrophoresis followed by Sephadex G-100 gel filtration.

Agarose-gel electrophoresis

The details of the procedure used for agarose-gel electrophoresis for purification have been described elsewhere (Natu & Chaturvedi, 1977). Briefly, 18 ml of 0·9% agarose dissolved in PBS, pH 7·0 was allowed to solidify on a glass slide measuring 11 × 8 cm. Wells cut at 1 cm distance were filled with CF tagged with bromphenol blue. The electrophoresis was carried out for 4 h at 4°, in a refrigerator, using constant voltage of 2 mA/cm of gel. One centimetre slices were ground in a mortar and pestle with 1 ml PBS to elute the proteins. The fractions were centrifuged and the clear supernatants were monitored by their absorption of ultraviolet light of 280 nm wavelength. The cytotoxic activity of the fractions was determined. In some of the preparations, human plasma was run on the lower portion of the same plate. The position of plasma proteins was determined by immune precipitin lines (Natu & Chaturvedi, 1977). The electrophoretic mobility of CF was compared with that of plasma proteins.

Gel filtration

Gel filtration of the cytotoxic factor purified by agarose-gel electrophoresis, was performed on a column of Sephadex G-100, 2·5 × 48 cm, eluting with PBS, pH 7·0 at 4°. Three-millilitre fractions were monitored by their absorption of ultraviolet light of 280 nm wavelength. Each fraction was screened for cytotoxic activity.

The approximate molecular weight of CF was determined by elution of other proteins of known molecular weight through the same column in several separate experiments (Andrews, 1964). The standard proteins were cytochrome C, chymotrypsinogen A, ovalbumin, bovine serum albumin, aldolase (Calibration Proteins, Ref. A-VZW/rt, Galenus GMbh), blue dextran and phenol red.

Effect of centrifugation of CF

The CF was ultracentrifuged at 30,000 r.p.m. for 3 h. The supernatant and the sediment were separated. The sedimented part was reconstituted with PBS to the original volume and the cytotoxic activity of both were studied.

Effect of Millipore filtration on CF

The CF was filtered through 0·45 μm Millex disposable filter units (Millipore Corporation, Bedford, Massachusetts, U.S.A.). The cytotoxic activity of the filtered and unfiltered cytotoxic factor was assayed.

Effect of dialysis on CF

One millilitre CF was dialysed against 4 ml PBS, pH 7·0, at 4° for 18 h. The dialysate and the contents of the dialysis bag were collected separately, and assayed for cytotoxic activity.

Effect of heat on CF

The CF was incubated in a water-bath for 30 min at temperatures ranging from 4 to 80°. It was then centrifuged at 3000 r.p.m. for 10 min at 4° to remove the precipitate, if any. The supernatant was assayed for cytotoxic activity.

Effect of pH on CF

The pH of the CF was adjusted from 1 to 10 with 1M HCl or NaOH and the solution was incubated for 30 min at room temperature. The pH was readjusted to 7·0 and the preparation was centrifuged at 3000 r.p.m. for 10 min at 4°.

Effect of trypsin on CF

1·5 mg of trypsin (Difco-250) was mixed with 1 ml CF and the pH of the solution was maintained at 7·0. After incubation at 37° for varying times between 15 min and 4 h, the preparations were centrifuged in the cold at 3000 r.p.m. for 10 min and crystalline soya bean trypsin inhibitor was added in excess to the supernatant to stop further enzyme activity.

Preparation of target spleen cells

Spleens were collected aseptically from normal healthy adult mice. The spleen cells were teased out.

in cold MEM supplemented with 5% foetal calf serum (Chaturvedi, Tandon & Mathur, 1978b). The single cell suspension was washed and the cells were treated with Tris–ammonium chloride pH 7·2 to remove erythrocytes. The cells were washed and viable nucleated cells were counted by the trypan blue dye exclusion test. Viability varied from 85 to 95% in different preparations.

Cytotoxicity test

The test for cytotoxicity was carried out in perspex trays, using 0·1 ml volumes of each preparation (Chaturvedi *et al.*, 1980). Briefly, various doubling dilutions of the preparations were added to a standard $2{\cdot}0 \times 10^6$ target cells. The plates were incubated at 4° for 1 h with frequent shaking. Viable cells were counted using trypan blue dye exclusion. All the tests were set up in duplicate and mean values of percentage non-viable cells are presented with SEM, obtained in replicate experiments (14–20 observations). The background non-viable cells were deducted from each value. The data were analysed by Student's *t test* for *P* values.

RESULTS

Agarose-gel electrophoresis of CF

The fractions obtained by electrophoresis were screened for proteins at 280 nm and for cytotoxic activity. Peak cytotoxic activity was present in fraction 5 which killed $41 \pm 2\%$ target cells while fraction 4 killed 9% and fraction 6 killed 7% target cells (Fig. 1). Thus the purest CF was obtained in fraction 5. It was further noted that on the basis of electrophoretic mobility, the CF occupied a position between α_1 and α_2 globulins.

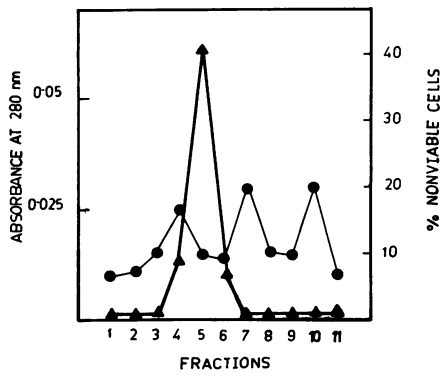

Figure 1. Prolonged agarose-gel electrophoresis of CF. The fractions were tested for proteins at 280 nm (●) and for cytotoxicity (▲) using normal mouse spleen cells as target. Fraction 5 was collected and used for further study.

Sephadex G-100 gel filtration of CF

Sephadex G-100 column chromatography of agarose-gel purified fraction (Fr. 5) of CF was performed. Figure 2 summarizes the results of tests for protein at 280 nm and for cytotoxicity on eluted

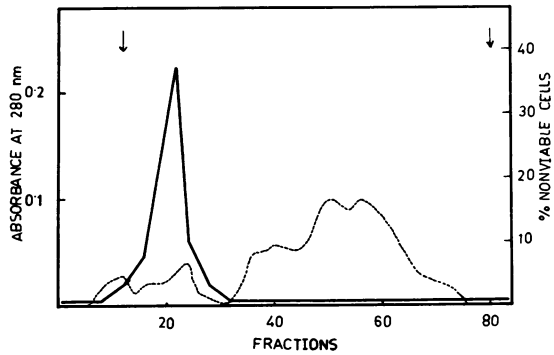

Figure 2. Sephadex G-100 gel chromatography of the fraction 5 of CF obtained by agarose-gel electrophoresis; 3 ml fractions were collected. Each fraction was screened for protein at 280 nm (– – –) and for cytotoxicity (—) against normal mouse spleen cells. The elution volumes of blue dextran and phenol red were determined and are indicated by vertical arrows.

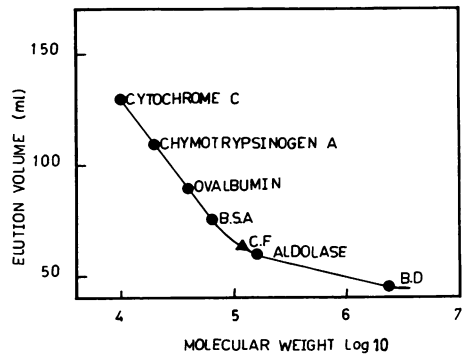

Figure 3. Estimation of molecular weight of the CF. After applying fraction 5 (1·5 ml) from agarose-gel electrophoresis each fraction was tested for cytotoxicity against normal mouse spleen cells. The relationship between elution volume and the logarithm of the molecular weights of various standard proteins was also established on this column and a curve was plotted. BD, blue dextran.

fractions. A single peak for CF was obtained with maximum cytotoxicity in fractions 21 and 22 which killed 37 and 36% target cells, respectively. The molecular weight of CF was estimated by calibrating the same column by eluting proteins of known molecular weight. A calibration curve was plotted of $\log_{10}$ molecular weight of marker protein versus elution volume according to the method of Andrews (1964). The point of maximum peak height of cytotoxic activity of CF was superimposed on the same curve. An approximate molecular weight of 1·15 ($\pm$0·34) $\times 10^5$ was calculated for the CF from the curve (Fig. 3).

Effect of heat on CF

The results shown in Fig. 4 indicate that the activity of CF was significantly reduced ($P < 0.001$) after 30 min of heating at 42° and virtually abolished at higher temperatures.

Effect of trypsin treatment on CF

Figure 5 presents the effects of trypsin treatment on CF. The cytotoxic activity was significantly reduced in 30 min ($P < 0.001$) and was completely abolished in 4 h.

Effect of pH on CF

Cytotoxic activity was significantly reduced ($P < 0.005$ to < 0.001) at acidic pH below 5 and at alkaline pH of 8 and above. Peak activity was seen at pH 7 (Fig. 6).

Effect of dialysis on CF

The dialysed fraction of CF killed 4·4 $\pm$ 2% spleen cells in contrast to 28 $\pm$ 3% cells killed by non-dialysable fraction.

Effect of Millipore filtration on CF

Findings presented in Table 1 show that CF does not

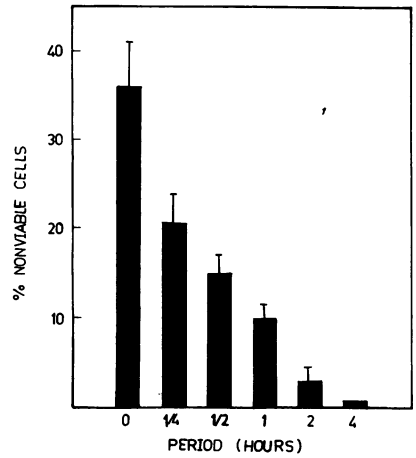

Figure 5. Effect of trypsin treatment on cytotoxicity of CF. Each column represents the mean value with sem 10–15 observations.

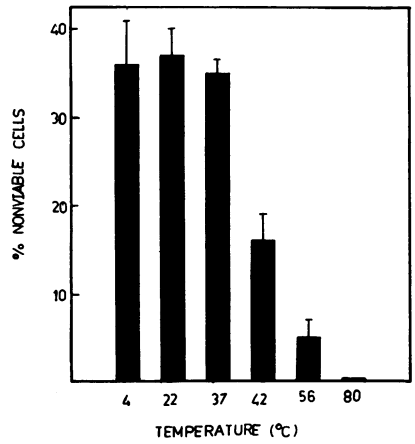

Figure 4. Effect of heat on cytotoxicity of CF. Each column represents mean value with standard error of the mean 10–15 observations.

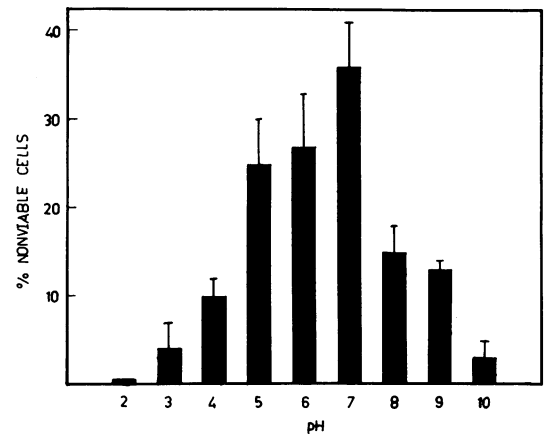

Figure 6. Effect of pH on the cytotoxicity of CF. Each column represents mean value with standard error of 8–15 observations.

Table 1. Comparison of CF and mouse lymphotoxin

	Mouse lymphotoxin*	Cytotoxic factor
Molecular weight	9×10^4 to $1{\cdot}5 \times 10^5$	$1{\cdot}15(\pm 0{\cdot}34) \times 10^5$
Effect of heat		
56° (30 min)	Stable	Inactivated
80° (30 min)	Stable	Inactivated
100° (15 min)	Stable	Inactivated
Effect of pH	Stable at pH 2–12	Unstable at pH 2–5 and 8–10
Effect of dialysis	NK	Non-dialysable
Effect of trypsin	Stable	Inactivated
Millipore Filtration	Filterable	Non-filterable

* Mainly from Pick & Turk (1972).
NK = not known.

pass through Millipore filter as the filtrate killed $1{\cdot}8 \pm 1{\cdot}4\%$ target cells in contrast to $36 \pm 5\%$ cells killed by unfiltered CF.

Effect of ultracentrifugation on CF

Centrifugation of CF at 30,000 r.p.m. for 3 h sedimented most of the cytotoxic activity; the supernatant killed $3{\cdot}4 \pm 2{\cdot}8\%$ target cells while the sedimented fraction killed $23 \pm 2\%$ cells.

DISCUSSION

The data presented here demonstrate that the spleen homogenate of DV-infected mice contains a cytotoxic factor which can be purified by agarose-gel electrophoresis and Séphadex G-100 column chromatography. It has been shown that CF is produced by T lymphocytes (Chaturvedi *et al.*, 1980). Different biological activities have been attributed to soluble substances released by lymphocytes and particularly by T cells. A product of T lymphocytes comparable with CF is lymphotoxin, a cytotoxic substance produced by cultured lymphoid cells stimulated by a mitogen or a specific antigen (Ruddle & Waksman, 1968; Jeffes & Granger, 1975).

As observed in an earlier study (Chaturvedi *et al.*, 1980), CF is similar to lymphotoxin in being secreted by lymphoid cells on stimulation by an antigen (DV); the activity is present both in the cells as well as being secreted. Many differences from the classical lymphotoxin have been observed, viz. stimulation is achieved *in vivo*; production *in vitro* does not require the addition of antigen; the target is specifically lymphoid cells of the spleen; and the action on target cells is effected in 1 h at 4° (Chaturvedi *et al.*, 1980). The findings in the present study confirm these differences and further show that CF is different from lymphotoxin in physicochemical properties also. A comparison of the properties of CF and mouse lymphotoxin given in Table 1 illustrates this point. A cytotoxin of human origin which is non-dialysable but inactivated at 56° has also been described by Lebowitz & Lawrence (1969).

The findings of several experiments show that the cytotoxic factor is a macromolecule viz. it can be sedimented by ultracentrifugation; it is non-dialysable, and it is retained by Millipore filters. The approximate molecular weight is $1{\cdot}15(\pm 0{\cdot}34) \times 10^5$. With the available data the possibility cannot be excluded that the cytotoxicity is due to a small molecule bound to a larger carrier or the small molecules of CF are aggregated together. We have observed aggregated CF particles when the sediment obtained after centrifugation at 30,000 r.p.m. is resuspended. The ability of the Millipore filter to retain the CF favours this view and is further supported by the finding of aggregation of lymphotoxin molecules *in vitro* (Boulos, Rosenau & Goldberg, 1974).

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