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Inhibition of E-rosette formation and phagocytosis by human blood leucocytes after treatment with the dengue virus-induced cytotoxic factor

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Summary. We have observed earlier that T lymphocytes of dengue type 2 virus (DV)-infected mouse spleen produce a cytotoxic factor (CF) which kills T lymphocytes and macrophages of the spleen of normal mice or animals of other species. In the present study an effort was made to study the effect of CF treatment on human peripheral blood leucocytes. After treatment with various dilutions of CF at 4° for 1 hr 25%–36% of T lymphocytes lost their capacity to form E rosettes and 25%–32% of monocytes lost their phagocytic function. Cytotoxic-factor treatment had no effect on formation of EAC rosettes by B lymphocytes and the phagocytic functions of polymorphonuclear cells. Pretreatment of cells with 2,4 dinitrophenol, reduced glutathione or ouabain, which act on the cell membrane, inhibited the effect of CF on E-rosette formation and phagocytosis. This indicated that CF acts by inducing changes in the cell membrane. It is likely that production of a similar factor in DV-infected humans is responsible for similar alterations observed in their blood.

INTRODUCTION

Changes in blood leucocytes have been observed during a number of viral infections of man and animals. Alterations that occur in peripheral blood leucocytes during dengue virus (DV) infection have been described only in a few reports. Simmons, Johns & Reynolds (1931) have shown leucopenia due to neutropenia while Nimmannitya, Halstead, Cohen & Margiotta (1969) and Halstead, Nimmanitya & Cohen (1970) have observed leucopenia associated with lymphocytosis. During an epidemic of dengue virus infection we have observed leucopenia in about 23% of cases and lymphocytosis in 31% of cases (Chaturvedi, Kapoor, Mathur, Chandra, Khan & Mehrota, 1970). Wells, Scott, Pavanada, Sathitsathein, Cheamudon & Macdermott (1980) have shown that in cases of dengue haemorrhagic fever, a significant lymphocytosis associated with the loss of T lymphocytes (as shown by SRBC rosettes) occurs with an increase in non-T, non-B, non-Fc receptor-bearing null cells. There was no change in the concentration of monocytes, B cells and Fc receptor-bearing cells. There are reports of depletion of T lymphocytes in patients dying of dengue haemorrhagic fever (Aung-Khin, Khin & Thant-Zin, 1975) and also in experimental animals (Tandon, Chaturvedi & Mathur, 1979). The pathogenesis of changes occurring in peripheral blood leucocytes or in those of lymphoid organs during dengue virus infection is not clear.

Recently we have observed that T lymphocytes of DV-infected mouse spleen produce a cytotoxic factor (CF) which kills lymphoid cells *in vitro*, mainly macrophages and some T cells, obtained from mice and a number of species of animals (Chaturvedi,

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Bhargava & Mathur, 1980a; Chaturvedi, Dalakoti & Mathur, 1980b; Chaturvedi, Mathur, Gulati & Mathur, 1981a; Chaturvedi, Shukla, Mathur & Mathur, 1981b). Inhibition of SRBC rosette formation by T lymphocytes of human peripheral blood can occur *in vitro* by treatment with serum obtained from patients of hepatitis virus infection containing a factor (Chisari & Edgington, 1975a) or by prostaglandins (Misefari, Venza-Teti & Lavi, 1980; Venza-Teti, Misefari, Sofo, Fimiani & Lavi, 1980). Since CF affects leucocytes of a number of species of animals, it was considered worthwhile to investigate its affect on some of the functions of human blood cells. The findings presented here demonstrate that CF inhibits functions of T lymphocytes and monocytes but has no effect on B lymphocytes and polymorphonuclear cells.

MATERIALS AND METHODS

Preparation of cytotoxic factor

The details of dengue type 2 virus and the preparation of CF from the DV-infected mouse spleen have been described elsewhere (Chaturvedi, Tandon & Mathur, 1977; Chaturvedi, Tandon & Mathur, 1978a; Chaturvedi, Tandon, Mathur & Kumar, 1978b; Chaturvedi *et al.*, 1980a,b).

Separation of blood cells

All the tests were done on blood collected from two persons (U.C.C. & L.G.). About 10 ml of heparinized venous blood was collected and was allowed to stand for 1 hr at room temperature. The leucocyte-rich plasma was separated, and 4 ml were carefully layered over 4 ml of Lymphoprep (sodium metrizoate and Ficoll) with a density of 1·077 g/ml (Nyegaard & Co. As., Oslo) in a 10 ml tube. It was centrifuged at 20° at 400 ***g*** for 30 min. The interface layer consisting of mononuclear cells and the bottom layer consisting of polymorphonuclear cells were collected separately. The mononuclear cell rich fraction contained 80%–90% lymphocytes and 10%–20% monocytes. The cells of both the fractions were washed three times with minimum essential medium (MEM). The viability was ascertained by the trypan blue dye exclusion method (Chaturvedi *et al.*, 1978a). The following tests were performed on the cells.

Sheep red blood cell rosettes

T lymphocytes are known to form spontaneous rosettes with sheep red blood cells (SRBC). One million mononuclear cells suspended in 0·1 ml were added to tubes containing 0·1 ml foetal calf serum. The foetal calf serum had been heat inactivated and absorbed with SRBC. To this was added one-fifth of a millilitre of 2% SRBC and the mixture was incubated at 37° for 10 min and then centrifuged at 200 ***g*** for 5 min and then incubated in an ice bath for 90 min. The pellet was gently resuspended and mixed with methylene blue to make a final concentration of 0·1% of the dye. Two hundred or more lymphocytes were counted and the percentage of rosette forming cells was determined. A rosette was defined as a lymphocyte binding three or more SRBC.

Antigen-antibody complement (EAC) rosettes

Modified technique of Bianco, Patrick & Nussenzweig (1970) was adopted for enumeration of B lymphocytes. SRBC were coated with anti-sheep haemolysin and normal human serum. One-fifth of a millilitre of lymphocyte suspension (2×10^6 cells/ml) was mixed with 0·2 ml of the coated SRBC. The tubes were kept at 37° for 90 min followed by centrifugation at a very slow speed. After incubation cells were gently resuspended and rosette forming lymphocytes were counted by mixing methylene blue dye as described above.

Neutral red dye test

This test was used to assess the phagocytic activity of the mononuclear cells as well as the polymorphonuclear cells (Cohn & Weiner, 1963). To 0·5 ml of cell suspension ($5–10 \times 10^6$ cells), 50 μl of 0·1% neutral red dye was added and incubated at 37° for 20 min with constant shaking. The cells were washed twice and resuspended in MEM. The percentage of cells containing neutral red granules was calculated after counting 200 or more cells.

Drug treatment of target cells

Effect of CF on target cells pretreated with 2,4 dinitrophenol (B.D.H. Chemicals, Ltd, Poole), reduced glutathione (Sarabhai M. Chemicals) and ouabain (E. Merck, Darmstadt) was studied. In another study we have screened a number of drugs for their inhibitory effect on the cytotoxicity of CF on mouse spleen cells. The above three drugs were found to be inhibitory (data to be published), therefore, in the present study we have used them. In preliminary experiments, various concentrations of the drugs were used and the one giving optimum results has been used in the test. The final dilution of 2,4 dinitrophenol

(DNP) in the cell treatment was 10^{-5} M, that of reduced glutathione was 10^{-4} and ouabain was used at concentrations of 10^{-3} M. The cells were treated with the drug at 4° for 1 hr, washed twice and then used as target cells. For control, CF was incubated with the drug at 4° for 1 hr and then its activity was assayed. This was done to check if the drug directly inactivates the CF. None of the three drugs had any effect on the cytotoxicity of the CF.

CF treatment of cells

The cells were divided into two aliquots, one was treated with CF at 4° for 1 hr and the second, used as control, was treated with the diluent. Then the cells were washed twice and screened for various functions described above. Normal mouse spleen homogenate (NH) was processed, similar to the preparation of CF, and has been used for treating the cells in place of CF in every test for control. All the tests were run in triplicate and repeated at least three times. The mean values with ± standard deviation have been presented. The data have been subjected to Student's *t* test for determination of *P* value.

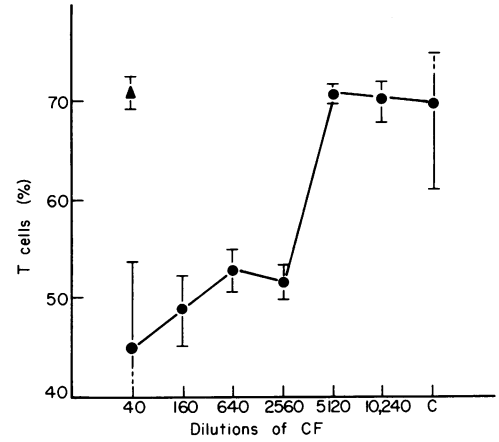

Figure 1. Effect of CF treatment on formation of SRBC rosettes by T lymphocytes. Lymphocyte-rich population obtained by centrifugation of leucocyte rich plasma on Lymphoprep was treated with various dilutions of CF at 4° for 1 hr and then tested for formation of E rosettes. (C) Cells treated with diluent in place of CF; (▲) cells treated with normal mouse spleen homogenate. Mean values ± SD presented.

RESULTS

Effect of CF treatment on viability of cells

The enriched populations of the leucocytes were washed and divided into two aliquots, one was treated with CF for 1 hr at 4° and the other was similarly treated with the diluent. The viability of the cells was assessed using trypan blue dye. It was observed that in different sets of experiments the non-viable cells, both in CF treated and untreated portions ranged from 2% to 4%. The same preparation of CF killed 33%–40% of mouse spleen cells.

Effect of CF treatment on E rosettes

The findings of the E rosette count in cells treated with various dilutions of the CF have been presented in Fig. 1. In the untreated controls 70% ± 5% of cells formed rosettes. In contrast, the count of E rosettes was 45% ± 8% to 53% ± 2% ($P < 0{\cdot}001$) in the cells treated with CF diluted up to 2560-fold. With higher dilutions of CF the rosetting was not affected.

Effect of CF treatment on EAC rosettes

It was observed that CF treatment of cells had no

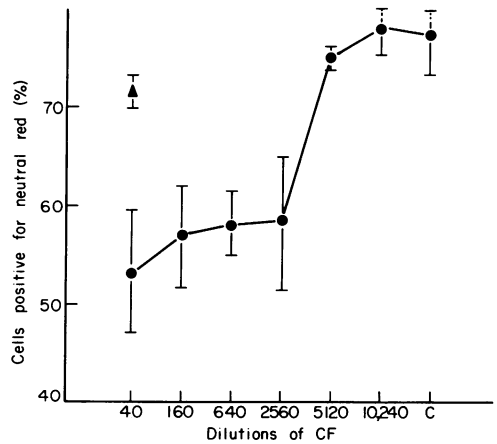

Figure 2. Effect of CF treatment on phagocytic activity of monocytes. Mononuclear cell-rich population obtained by centrifugation of leucocyte-rich plasma on Lymphoprep was treated with various dilutions of CF at 4° for 1 hr and then tested for phagocytic activity by uptake of neutral red. (C) Cells treated with diluent in place of CF; (▲) cells treated with normal mouse spleen homogenate. Mean values with ± SD presented.

effect on formation of EAC rosettes. The count of EAC rosettes in untreated cells was 16%±1·3% while that in CF-treated fraction was 15%±1·4% ($P > 0{\cdot}200$).

Effect of CF treatment on phagocytosis by polymorphonuclear cells

The phagocytic function of polymorphonuclear cells was studied by the uptake of the neutral red dye. The findings show that CF treatment had no effect on uptake of the dye. Among the CF-treated sets 79%±7% of cells had taken up the dye while 83%±5% untreated cells showed neutral red granules, the difference being insignificant ($P > 0{\cdot}200$).

Effect of CF treatment on phagocytosis by mononuclear cells

The uptake of neutral red by fractions rich in mononuclear cells is summarized in Fig. 2. Cells positive for neutral red in the untreated control sets were 77%±4%. Among the cells treated with CF diluted forty- to 2560-fold, 53%±5·7% to 58%±7% cells had taken up the dye. The inhibition of phagocytosis by CF treatment was highly significant ($P < 0{\cdot}001$). Percentage of cells taking up the dye after treatment with higher CF dilutions was similar to that in controls (Fig. 2).

Drug treatment inhibits the effect of CF on E-rosette formation by T cells

Efforts were made to gain some insight into the biochemical mechanism by which CF kills the target cells. For this, we have used some chemical substances, which have known effects on the cells, to inhibit the action of CF. Data presented in Fig. 3 indicate that in untreated or NH-treated controls, the E-rosette-forming cells were 77%±3·4% and 71%±2·2% respectively. In contrast the reduction in E rosettes in CF-treated cells was 47% ($P < 0{\cdot}001$). CF had almost no effect on E rosettes if the cells were pretreated by the drugs (Fig. 3). Further, the drug treatment *per se* had no effect on rosette formation (data not presented).

Drug treatment inhibits the effect of CF on the phagocytosis by mononuclear cells

Findings summarized in Fig. 4 show that the phagocytosis of the neutral red granules was reduced by 33% by incubating the cells with CF ($P < 0{\cdot}001$). The effect of CF was abolished if the cells were pretreated with the drugs. In the controls, the drug treatment *per se* had no effect on the phagocytosis (data not shown here).

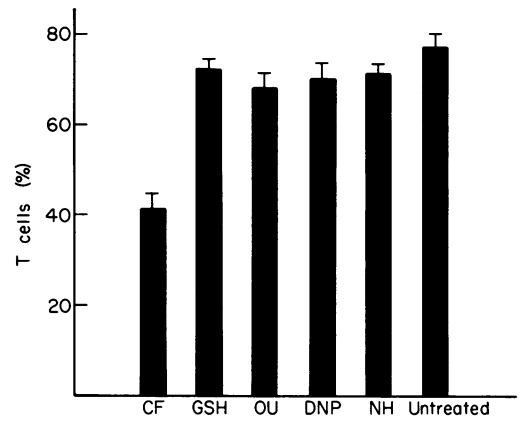

Figure 3. Drug treatment inhibits the effect of CF on E-rosette formation by T cells. Lymphocyte-rich cell population was incubated with CF after treatment with reduced glutathione (GSH), ouabain (OU) or 2,4 dinitrophenol (DNP) or without drug treatment (CF). Then E-rosette formation was studied. Normal mouse spleen homogenate (NH) treated or untreated cells were used as control. Mean values ± SD have been presented.

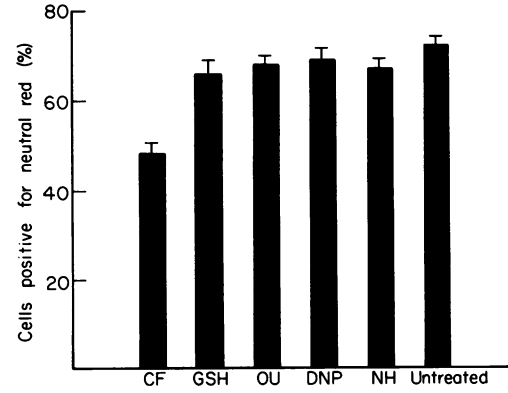

Figure 4. Drug treatment inhibits the effect of CF on phagocytosis by monocytes. Mononuclear cell-rich population was incubated with CF after treatment with reduced glutathione (GSH), Ouabain (OU) or 2,4 dinitrophenol (DNP) or without drug treatment (CF). Then cells were tested for phagocytic activity by uptake of neutral red. Normal mouse spleen homogenate (NH) treated or untreated cells were used as control. Mean values ± SD have been presented.

DISCUSSION

DV-induced CF kills most of the macrophages and about one third of T lymphocytes obtained from the lymphoid organs of a number of species of animals but had no significant killing effect on their blood leucocytes (Chaturvedi *et al.*, 1981a). The data presented here indicate that CF does not kill the human peripheral blood leucocytes but has a definite effect on certain functions of T lymphocytes and monocytes.

One of the functions affected was formation of E rosettes by the T cells. After treatment with CF 25%–36% of T cells lost the capacity to form E rosettes. Further, the effect is produced on a subpopulation of T lymphocytes as all the cells did not lose the capacity to form E rosettes. This is similar to *in vitro* effect of CF on mouse spleen cells, killing one third of the T lymphocytes (Chaturvedi *et al.*, 1981a). In patients with DV-infection, E rosettes are reduced and the null cells are increased but total lymphocyte count remains unaffected (Wells *et al.*, 1980). On the basis of the findings of the present study it can be suggested that the T lymphocytes which have lost their capacity to form E rosettes will appear null cells in the test. It is tempting to speculate that a factor similar to mouse CF is produced in DV-infected humans and affects the functions of leucocytes including formation of E rosettes. It appears that some of the CF leaks out into the blood from the site of production. The findings of cytotoxic activity in the serum of some of the DV-infected mice support this assumption (Chaturvedi *et al.*, 1980a). An attempt may be made to screen the sera of patients having DV infection for the presence of CF. With more sensitive methods it may be possible to detect CF in greater numbers of sera than observed in our study earlier (Chaturvedi *et al.*, 1980a) and also in man.

In many disease states a reduction in the percentage of cells forming E rosettes have been noted *viz.* viral infections and cancer (Wybran & Fudenberg, 1973) and autoimmune diseases (Messner, Lindstrom & Williams, 1973). In some of these conditions a rosette inhibitory factor has been demonstrated in the serum (Chisari & Edgington, 1975a). It is, therefore, logical to conclude that test of E rosettes is not a good indicator of absolute number of T lymphocytes in peripheral blood during disease states. It is known that though E rosettes are reduced in number but some of the T-cell-dependent functions remain unaffected in such patients (Chaturvedi, Mathur, Tandon, Natu, Rajvanshi & Tandon, 1979).

The two sets of cells responsible for the phagocytic activity are the polymorphonuclear cells and the monocytes. It was observed that the uptake of neutral red was similar in CF treated or untreated polymorphonuclear cells. On the other hand the phagocytic activity of the monocytes was reduced by 25% to 32% ($P < 0{\cdot}001$) by treatment with various dilutions of CF.

The phagocytic activity depends upon intact glycolytic pathway of the cells. Reduced glycolysis has been shown to be associated with inhibition of phagocytosis during influenza virus infection (Fisher & Ginsberg, 1956; Sawyer, 1969). On the other hand many suggestions have been made regarding formation of E rosettes *viz.* it is a dynamic energy-dependent function (Bentwich, Douglas, Siegal & Kunkel, 1973); it is modulated by intracellular cyclic nucleotide levels (Chisari & Edgington, 1974; Galant & Remo, 1975); it needs intact glycolytic (Jondal, Holm & Wigzell, 1972), protein and nucleic acid (Bushkin, Pantic & Incef, 1974) synthetic pathways. Thus formation of E rosettes and the phagocytosis depends upon active metabolic functions of the cell.

The precise mechanism of killing of cells by CF as observed earlier (Chaturvedi *et al.*, 1980a, 1981a) and the induction of functional changes as observed in the present study is not known. Which of the various pathways are affected by CF is not known. The cells are not killed by the CF treatment, therefore, changes appear to be occurring at the functional level. It has been reported that such functional changes induced by the rosette inhibitory factor found in cases of viral hepatitis are reversible (Chisari & Edgington, 1975). We have noted that the activity of CF is not reduced after adsorption onto the susceptible or non-susceptible cells (Chaturvedi *et al.*, 1981a). This excludes the role through blockage of receptors by CF. It appears that CF induces certain changes in the cells which inhibit rosette formation by T cells and phagocytosis by monocytes. Since the changes are brought about so quickly, role of disturbed metabolism of the cells appear most likely. This is supported by the findings of inhibition of the effect of CF on drug-treated cells. DNP enhances the conductance of the phospholipid bilayer of the cell resulting in increased transport of H^+ and Na^+ ions and some anions. It prevents the phosphorylation of ADP to ATP and produces a negative surface potential (Thompson & Lehninger, 1966; Mitchell, 1966; Lea & Groghan, 1969). Reduced glutathione provides free SH groups and acts as activator of certain enzymes and in the protection of lipid membranes. While ouabain inhibits the Na^+-K^+

ATPase in the plasma membrane thus causing its depolarization (Hülser, Ristow, Webb, Pachowsky & Frank, 1974). Inhibition of cytotoxicity of the CF by pretreatment with these drugs indicate that CF induces changes in the functions of cell membrane which may cause ionic imbalance resulting in cell death. Further studies are needed to clarify this point.

Findings of the present study thus demonstrate that CF produced during DV infection can affect functions of peripheral blood leucocytes and an effort should be made to look for the presence of a similar factor in patients with DV infection.

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