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Dengue virus-induced human cytotoxic factor: production by peripheral blood leucocytes *in vitro*

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SUMMARY

During dengue type 2 virus (DV) infection of mice a unique cytokine, the cytotoxic factor (CF), is produced which reproduces the pathological lesions seen in patients of dengue haemorrhagic fever (DHF). Recently we have observed a CF-like protein in the sera of DHF cases. The present study was undertaken to investigate whether DV can stimulate human peripheral blood mononuclear cells (PBMC) *in vitro* to produce human CF (hCF). Cultures prepared from PBMC or its enriched subpopulations were inoculated with 1000 LD_{50} of DV and controls with normal mouse brain suspension (NMB). Aliquots of cultures were harvested daily from 24 h to 96 h and their supernatant (CS) and cells were separated. CS were screened for viral titres and for the presence of hCF by cytotoxicity assay, inhibition ELISA, dot blot and Western blot tests using anti-mouse-CF antibodies. The RNA from the cells was screened in Northern blot and dot blot tests for the presence of mRNA for CF. It was observed that hCF appeared in the CS of DV-infected culture of PBMC and T-enriched cells at 48 h and was present until 96 h; no CF was detected in CS of B cells or monocyte cultures. The production of hCF was abrogated by pretreatment of the T cells with anti-CD4 antibodies but not with anti-CD8 antibodies, indicating that hCF was produced by $CD4^+$ T cells. The Northern blot analysis using oligonucleotide probes prepared on the basis of amino-terminal sequence of mouse CF, showed presence of mRNA for hCF in PBMC and T cell cultures. DV replicated in PBMC cultures with peak titres at 48 h. The findings of the present study demonstrate that DV-induced hCF is produced by human T cells.

Keywords dengue virus cytotoxic cytokine human cytotoxic factor

INTRODUCTION

Increased vascular permeability, thrombocytopenia, cerebral oedema and alterations in blood leucocytes are the cardinal features of dengue haemorrhagic fever/shock syndrome (DHF/DSS). The pathogenesis of the disease is not fully known. A unique cytokine, the cytotoxic factor (CF) is produced by the T lymphocytes of dengue type 2 virus (DV)-infected mouse spleen [1]. It has recently been shown that CF is a pathogenesis-associated protein, capable of reproducing all the pathological lesions in mice that are seen in cases of human dengue [2–4]. CF is a molecule of 22–25 kD on SDS–PAGE with an isoelectric point of pH 6·5. The N-terminal sequence of 19 amino acids of CF does not match with any other known cytokine or dengue virus-specific proteins [5]. Oligonucleotide probes derived from this sequence show the presence of mRNA for CF in the spleen cells of DV-infected mice by Northern blot test. The present study has been planned to investigate if human peripheral blood leucocytes can be stimulated by DV *in vitro* to produce human CF (hCF). This is the first study to describe production of DV-induced CF by human $CD4^+$ T cells.

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MATERIALS AND METHODS

Mice

The study was carried out on inbred Swiss albino mice aged 2–3 months, obtained from the colony maintained in this Department.

Virus

Dengue type 2 virus (DV), strain P23085, obtained from the National Institute of Virology (Pune, India), was used in the form of infected mouse brain suspension [6]. Similarly prepared normal mouse brain homogenate (NMB) was used in controls. Virus was titrated by intracerebral (i.c.) inoculation of groups of mice in serial 10-fold dilutions of the supernatants of peripheral blood mononuclear cell (PBMC) cultures collected at different times post-infection. The virus titre was calculated

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by the method of Reed & Muench [7] and expressed as $\log_{10}$ LD_{50} per millilitre of the culture supernatant.

Preparation of PBMC

Peripheral venous blood was collected from the authors (R.M. and U.C.C. alternately) in a heparinized tube. The leucocyte-rich plasma was removed and the PBMC consisting of monocytes and lymphocytes were separated as described earlier [8]. Briefly, the leucocyte-rich plasma was layered over Lymphoprep, density 1·077 g/ml (Nyegaard & Co. As., Oslo, Norway) and centrifuged at room temperature at 400 ***g*** for 30 min. The interface layer was collected, and the cells were washed three times with Hanks' basal salt solution (HBSS). The cells were counted and smears were stained with Giemsa stain to study cell morphology.

Preparation of enriched population of T and B cells

Washed PBMC were passed through glass wool columns to remove monocytes and were then filtered through nylon wool columns to obtain an enriched subpopulation of T and B cells [9]. The cells were counted and 1×10^6 cells/ml were cultured for different assay.

Preparation of monocyte-enriched cells

The PBMC were suspended in Eagle's minimum essential medium (MEM) containing 5% fetal calf serum (FCS), layered in 4-cm Petri dishes (Nunclon, Nunc, Denmark) and incubated at 37°C in an atmosphere of 5% CO_2 in air. After 2 h the Petri dishes were shaken and the non-adherent cells decanted. The process was repeated three times using fresh HBSS every time. Most of the glass-adherent cells were phagocytic, as shown by phagocytosis of latex particles [8]. These cells were therefore considered as monocytes in the present study.

Treatment of T cells with CD antibodies and complement

The enriched T cells were treated with mouse anti-human CD4 or CD8 MoAbs (Dako A/S, Glostrup, Denmark) and rabbit complement by the technique of Liew & Simpson [10]. The cells were washed three times and their viability estimated by trypan blue dye exclusion. The cells were counted and cultured. One set of cultures was inoculated with DV and the controls with NMB. The cultures were harvested at 24, 48, 72 and 96 h and the culture supernatants (CS) and cells were collected for screening for the presence of hCF.

Preparation of cell cultures

The PBMC or its enriched subpopulation of cells (1×10^6 cell/ml) were cultured in MEM containing 5% FCS using 4-cm Petri dishes (Nunclon; Nunc). The cultures were inoculated with 1000 LD_{50} of DV, and the control cultures with NMB; tubes were incubated at 37°C in the presence of 5% CO_2 for various time periods. All the cultures were set in triplicate and the experiments repeated at least three times. CS were tested for the presence of hCF by cytotoxicity, ELISA and dot blot assay. The cells were processed to isolate total RNA for Northern blot test to screen the presence of hCF-specific mRNA.

Assay of cytotoxic activity

The cytotoxic activity of preparations was assayed using normal mouse spleen cells as target. Spleen cells were teased out in cold MEM to make a single-cell suspension and nucleated viable cells were counted by the trypan blue dye exclusion test. The technique for the assay of cytotoxic activity of CF is described elsewhere [1]. Briefly, 100 μl of the test solution were mixed with 100 μl of target cells (2×10^6 cells) in 96-well perspex plate and kept at 4°C for 1 h. Viability of cells was assayed by trypan blue dye exclusion and the percentage of non-viable cells was calculated. The data were presented after deduction of the background values obtained from uninoculated controls.

ELISA

The technique of Voller *et al.* [11] was used with some modifications to set up ELISA for detection of CF (Mukerjee *et al.*, in preparation). Briefly, a polystyrene flat-bottomed microtitre plate (Titertek Immuno assay plate 77-173-05; Flow Laboratories, Zwanenburg, The Netherlands) was coated with 1:5000 dilution of mouse CF-specific antibody [3,4] in PBS overnight at 4°C. The plate was washed three times with PBS pH 7·2 containing 0·05% Tween-20 (PBS–T) and blocked with 1% milk protein (Lactogen-1 milk powder; Nestle India Ltd, New Delhi, India) in PBS for 1 h. The plate was washed again three times with PBS–T, and 100 μl of the test samples were added and the plate was incubated at 37°C for 1 h. After washing of the plate, 100 μl of protein-A conjugated with horseradish peroxidase (HRP; a gift from the National Institute of Immunology, New Delhi, India) diluted 1:10 000 in dilution buffer (blocking buffer plus 0·5% Tween-20) were added and incubated at 37°C for 1 h. The plate was washed three times and colour was developed by addition of 100 μl of mixture of *o*-phenylene diamine (OPD; Sigma Chemical Co., St Louis, MO) and H_2O_2 in citrate buffer (0·02 M) pH 5·0. The plate was incubated at room temperature for about 10 min and reaction was stopped with 50 μl of 2·5 N H_2SO_4 and absorbance was read at 492 nm in an Automated Microplate Reader (EL 311sx; Bio-Tek Instruments Inc., USA). The following controls were included: (i) known mouse CF as positive control; (ii) a heterologous cytokine (suppressor cytokine) as negative control. The optical density (OD) from wells coated with the antibody without addition of any antigen was considered as 100% binding of the protein A + HRP, while wells without antibody coating yielded values for the blank. hCF present in the test sample bound with the antibody on the solid phase and inhibited subsequent binding of the protein A + HRP, thus indicating presence of hCF. The percentage inhibition was calculated as follows: per cent inhibition = 100 – (OD with test samples – OD of blank)/(OD without test sample – OD of blank) × 100.

Immunoblotting

Culture supernatants were blotted on nitrocellulose (NC) paper in a Milliblot System (Millipore Corporation, Bedford, MA). Some of the representative samples were resolved on 12·5% polyacrylamide gel at a constant current of 30 mA in a vertical slab gel system (Pharmacia, Uppsala, Sweden) and electroblotted (Novablot Electrophoretic Transfer Kit; Pharmacia) onto NC paper. The blots were air-dried and blocked overnight at 4°C in 3% bovine serum albumin (BSA) prepared in 0·1 M Tris HCl pH 7·5. After three washings with PBS–T, NC paper was incubated with mouse CF-specific antibody (1:500 dilution in PBS–T) for 1 h on a rocking platform at room temperature. The NC paper was washed again three times with PBS–T and incubated with anti-mouse IgG linked to HRP (1:500 in PBS–

T) for 1 h at room temperature. After extensive washing the blots were developed using diamino benzidine (Sigma) and hydrogen peroxide. For control the blots were treated with anti-DV antisera or normal mouse sera in place of anti-CF antisera.

End-labelling of oligonucleotides

The two oligomers used were: 5′ TGG CTC TAG ACA 3′ and 5′ TGG TTC TAG ACA 3′. The sequence of the above oligomers was chosen from the N′-terminal sequence of mouse CF [5] and was prepared by CSIR Biochemical Biotechnology (New Delhi, India). To 1 μl of the oligomer was added 10 × kinase buffer containing cocktail of dNTPs, T4-polynucleotide kinase, water and 30 μCi ^{32}P-γATP, final volume 10 μl [13]. The mixture was incubated at 37°C for 30 min and then loaded on a Sephadex G25 column. The labelled oligonucleotides were recovered from the column with water and tested for specific radioactivity.

RNA isolation and Northern blot analysis

At different time periods after exposure of leucocytes, T lymphocytes and B lymphocytes from human blood to DV, total RNA was isolated by acid guanidinium-thiocyanate-phenol-chloroform extraction method, as described by Chomczynski & Sacchi [12]. The RNA was quantified spectrophotometrically and its integrity examined by electrophoresis.

For Northern blot analysis, 20 μg of total RNA per lane from each group was applied and separated by electrophoresis on 1% agarose gel under denaturing conditions [13]. The RNA was then transferred to NC membrane using the LKB Vaccugene blotting system. RNA was immobilized on NC membrane by vacuum baking at 80°C for 2 h. The blots were prehybridized for 4 h at room temperature in presence of saline sodium citrate buffer (SSC, 5 ×), 5 × Denhardt's solution, 1 mM EDTA, and were subsequently hybridized in the same mixture containing ^{32}P-labelled oligonucleotides for 15 h at 42°C under gentle shaking [13,14]. After hybridization, the NC membrane was washed three times for 10 min at 42°C in 2 × SSC and 0·2% SDS, and three times for 20 min in 0·1 × SSC and 0·1% SDS. The NC membrane was exposed at −70°C to Kodak x-ray film with intensifying screen. Autoradiograms were developed 7–10 days after exposure. For dot blot analysis 5 μg of total RNA were blotted directly onto NC membrane in a Milliblot System (Millipore Corporation) and hybridized as described above.

Statistical analysis

Data were analysed using Student's t-test. $P < 0.05$ was considered significant.

RESULTS

Detection of hCF by cytotoxic activity

A peak cytotoxicity of 39 ± 7% was observed in the CS collected at 48 h following DV inoculation in PBMC cultures. At later periods, activity was slightly reduced ($P > 0.05$). A peak activity of 7 ± 2% was observed in culture inoculated with NMB (Fig. 1a). Activity in CS from enriched DV-inoculated T cell was little less, maximum being 29 ± 5% (Fig. 1b), while CS from B cells or monocyte-enriched cultures had no cytotoxic activity (Fig. 1c,d).

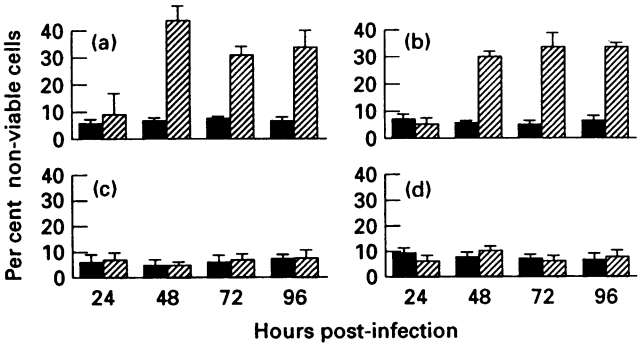


Fig. 1. Cytotoxic effect of culture supernatants (CS) from human peripheral blood mononuclear cells (PBMC) stimulated by dengue virus (DV) *in vitro* at different periods after virus infection. Cultures (1 × 10^6 cells/ml) were prepared in 4-cm Nunc Petri dishes of PBMC (a) or its enriched subpopulations of T cells (b), B cells (c) or monocytes (d). One set of cultures was inoculated with DV (▨), the second set with normal mouse brain homogenate (NMB) (■) and the uninoculated cultures were put up for blank. All cultures were incubated at 37°C in 5% CO_2 and harvested at different periods. The CS were assayed for cytotoxic activity using single-cell suspension of normal mouse spleen. The cells made non-viable after 1 h incubation at 4°C were counted using trypan blue dye exclusion. The results have been presented, after deduction of background values, as mean ± s.d. from nine to 15 observations for each group.

Detection of hCF by ELISA

An inhibition ELISA done to detect the presence of CF in CS of DV-inoculated cultures showed a peak inhibition of 48 ± 5% at 48 h in PBMC (Fig. 2a). A similar pattern was seen with CS from T cell-enriched cultures (Fig. 2b), while CS from B cells or monocyte-enriched cultures had insignificant ($P > 0.5$) inhibitory activity (Fig. 2c,d).

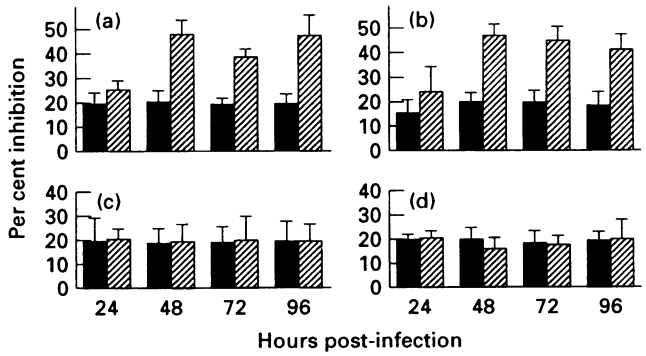


Fig. 2. Inhibition ELISA to detect the presence of cytotoxic factor (CF) in culture supernatants (CS) from peripheral blood mononuclear cell (PBMC) cultures. The groups of cells and the symbols are as in Fig. 1. The plate coated with anti-CF antibody was incubated with CS from different groups. After washing it was incubated with protein A conjugated with horseradish peroxidase (HRP), followed by addition of *o*-phenylenediamine. OD was taken at 492 nm. Controls included mouse CF (positive control), a heterologous protein (negative control). Inhibition of the binding of protein A + HRP by the presence of CF in the CS was calculated from OD in the wells to which no protein was added, as described in Materials and Methods.

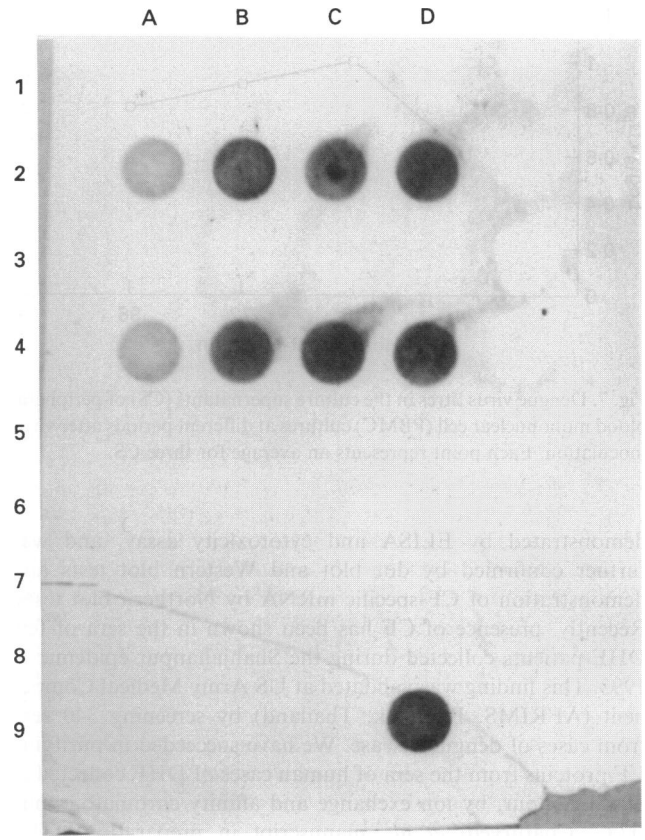


Fig. 3. Dot blot test for detection of cytotoxic factor (CF) in culture supernatants (CS) of the cultures of peripheral blood mononuclear cells (PBMC) or its enriched cell subpopulation as described in Fig. 1. CS were blotted on nitrocellulose paper. The blots were treated with anti-CF antibody followed by anti-mouse IgG + horseradish peroxidase (HRP) and then the substrate diamino benzidine as described in Materials and Methods. Some of the representative reactions are shown at 24 h (row A), 48 h (row B), 72 h (row C) and 96 h (row D) after infection; negative control (lane 9, B,C) and positive control mouse CF (lane 9, D); CS from dengue virus (DV)-inoculated T cells (lane 2), PBMC (lane 4), B cells (lane 6), monocytes (lane 8); CS from normal mouse brain homogenate (NMB)-inoculated culture of T cells (lane 1), PBMC (lane 3), B cells (lane 5) and monocytes (lane 7).

Detection of hCF by immunoblotting

All the CS collected from different experiments were screened in a dot blot test using anti-CF antibody. It was observed that CS from DV-stimulated cultures of PBMC or enriched T cells at 48–96 h reacted positively, while the reaction was negligible at 24 h. CS from controls, B cells and monocyte-enriched cultures did not show any reaction. Some of the representative reactions are shown in Fig. 3. The data presented in Fig. 4 show the reaction of the representative samples of each group in a Western blot test. The anti-CF antibody reacted with mouse CF (lane 1) and CS from DV-stimulated T cells (lane 5) and PBMC (lane 7) cells, but not with those of B cells (lane 3) and controls. Further, the migration of human CF was similar to that of mouse CF (Fig. 4).

hCF production by antibody-treated cells

The findings presented in Fig. 5 show that production of hCF was abolished by pretreatment of the T-enriched cells with anti-

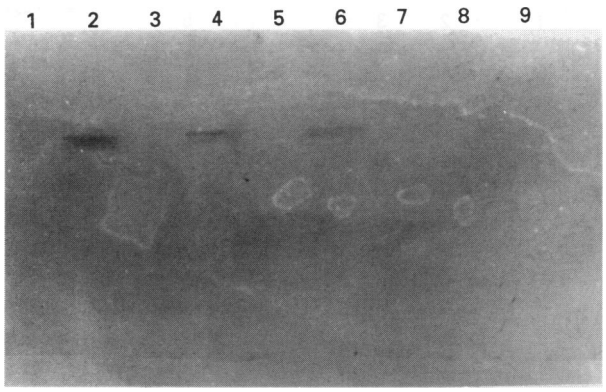


Fig. 4. Western blot analysis of some of the representative samples of culture supernatants (CS; as in Fig. 1) collected 48 h after dengue virus (DV) infection or normal mouse brain homogenate (NMB) inoculation. The samples resolved on PAGE were blotted on nitrocellulose paper and the blots were treated as in Fig. 3. The samples included were negative control (lane 1), pure mouse cytotoxic factor (CF) (lane 2), NMB-inoculated T cells (lane 3), DV-inoculated T cells (lane 4), NMB-inoculated peripheral blood mononuclear cells (PBMC) (lane 5), DV-inoculated PBMC (lane 6), NMB-inoculated PBMC (lane 7), DV-inoculated B cells (lane 8), DV-inoculated monocytes (lane 9).

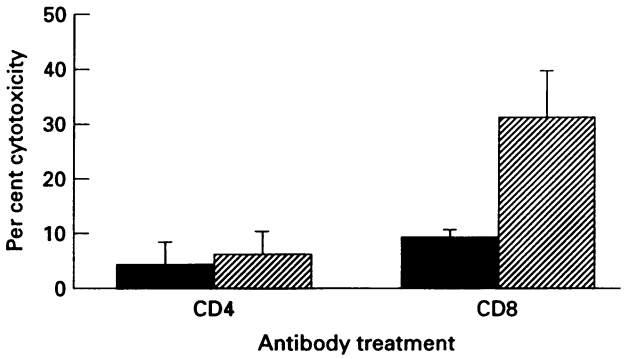
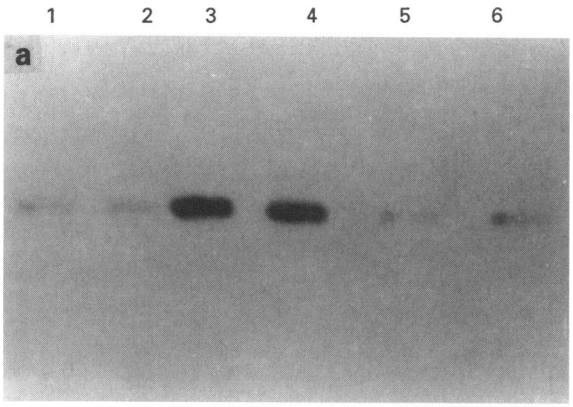


Fig. 5. Cytotoxic effect of culture supernatants (CS) from T-enriched cells pretreated with anti-CD4 or anti-CD8 antibody and complement. After washing, one set of the cells was inoculated with dengue virus (DV) (▨), the second set with normal mouse brain homogenate (NMB) (■) and the third set was an uninoculated control for blank. The cultures were incubated at 37°C in presence of 5% CO_2 for 48 h and the CS were collected. Cytotoxic activity of the CS was assayed as in Fig. 1. The mean value ± s.d. from 15 observations of each sample is presented.

CD4 antibodies as shown by cytotoxicity assay (6 ± 2%). Treatment with anti-CD8 antibodies had no effect on the production of hCF (cytotoxicity 31 ± 7%). Similar findings were observed when the CS from this experiment was assayed by ELISA for the presence of hCF (data not shown).

Detection of mRNA for hCF

Autoradiograms of the Northern blot and dot blot analysis of mRNA for hCF are presented in Fig. 6a,b. A positive reaction with CF-specific oligonucleotide probe was observed with DV-stimulated PBMC and T cells, but not with B cells. A faint reaction was observed with control T cells and PBMC (Fig. 6a).



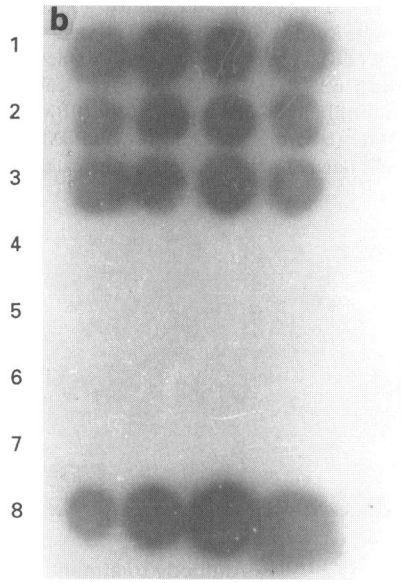


Fig. 6 (a) Northern blot analysis of total RNA isolated from dengue virus (DV)-infected T cells (lane 3) and peripheral blood mononuclear cell (PBMC) (lane 4) cultures; normal mouse brain homogenate (NMB)-inoculated T cells (lanes 1,2) and also PBMC (lane 5,6) cultures. Hybridization was done as described in Materials and Methods after size separation of 20 μg RNA on agarose gel and then blotting on nitrocellulose (NC) membrane. (b) Dot blot analysis of total RNA isolated from DV-infected T cells (lanes 1,2); PBMC (lane 3); B cells (lanes 4,5) and monocyte (lane 6,7) cultures. For controls RNA isolated from DV-infected mouse spleen was used as positive control (lane 8). Each spot represents 5 μg of RNA from one culture or one mouse spleen. Hybridization was done as described in Materials and Methods.

Detection of DV replication in MNC cultures

The average titres of the virus in the supernatants of three PBMC cultures at different time periods are shown in Fig. 7. The virus titre was highest at 48 h after DV infection.

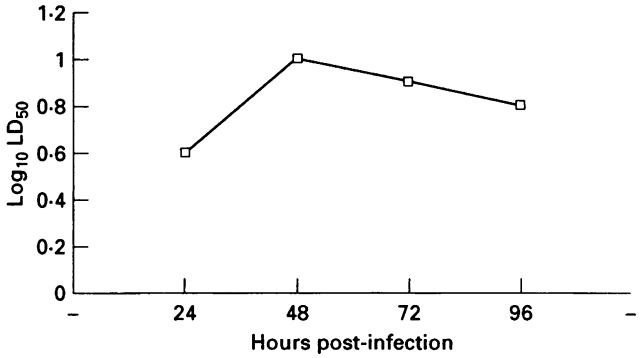


Fig. 7. Dengue virus titres in the culture supernatants (CS) of peripheral blood mononuclear cell (PBMC) cultures at different periods after virus inoculation. Each point represents an average for three CS.

DISCUSSION

The significant finding of the present study is that human T cells are capable of producing hCF on stimulation by DV *in vitro*. The presence of hCF in CS of DV-stimulated cultures was demonstrated by ELISA and cytotoxicity assay, and was further confirmed by dot blot and Western blot tests and demonstration of CF-specific mRNA by Northern blot tests. Recently, presence of CF has been shown in the sera of few DHF patients collected during the Shahjahanpur epidemic in 1993. This finding was validated at US Army Medical Component (AFRIMS, Bangkok, Thailand) by screening 340 sera from cases of dengue disease. We have succeeded in purifying CF proteins from the sera of human cases of DHF collected at Shahjahanpur, by ion exchange and affinity chromatography (U.C. Chaturvedi *et al.*, manuscript in preparation). This indicated that a protein which is pathogenesis-related in mice is also present in human cases and may explain some aspects of pathogenesis of the human disease.

An effort was made to identify the cell type in human peripheral blood leucocytes, responsible for the production of CF on stimulation by DV. It was observed that T cells were responsible for the production of hCF. The findings of the following experiments supported this conclusion on the basis of ELISA and cytotoxicity assay: (i) CF activity was similar in the CS from T cell-enriched (monocyte- and B cell-depleted) and PBMC subpopulation ($P > 0{\cdot}05$); (ii) CS from B cell-enriched population had no activity; (iii) CS from monocyte-enriched population had no hCF activity. These findings were confirmed by dot blot test also. Treatment of T cells with anti-CD4 antibody and complement abrogated the production of hCF, while treatment with anti-CD8 antibody and complement had no effect. This strongly suggested that hCF was produced by $CD4^+$ T cells. This is similar to the mouse CF which is produced by $L3T4^+$ splenic T cells ([1,15], unpublished data). Further, the expression of mRNA in PBMC and T cells reflected synthesis of hCF by the cells following stimulation by DV. Detection of a cytokine by expression of mRNA is a much more sensitive and specific technique, and has been used in PBMC by *in situ* hybridization [16].

The virus antigen is detectable in the spleen of mice, inoculated intracerebrally with DV by immunofluorescence from day 4 and the titre of the virus reaches peak levels from day 8 [17]. The CF is detectable in the spleens of such mice from day 6, reaching peak cytotoxic activity on days 10–11 [1]. In the present study DV was detectable throughout the period of study, with peak virus titres at 48 h, and the hCF was detected at 48 h after DV inoculation in the cultures and persisted to

similar levels up to hour 96 of the study. On the other hand, PBMC cultures inoculated with heat-killed DV showed neither the virus nor the production of hCF (data not shown). A similar time schedule is observed when mouse spleen cell cultures are infected with DV (unpublished data). DV has been shown to replicate *in vitro* in B-type lymphoblastoid cells; in mitogen-stimulated peripheral blood lymphocytes; in macrophages; and possibly in specifically sensitized lymphocytes (reviewed in [18]). It has been observed that the presence of macrophages is obligatory for the presentation of DV antigen to B cells, leading to their clonal expansion and consequent immune response. The B cells as such are incapable of receiving the native DV antigen [19,20]. The findings of the present study show that induction of hCF production by DV does not require any accessory cells; DV could directly stimulate T cells to produce hCF (Fig. 1b, 2b).

Mouse CF is a pathogenesis-associated protein and is capable of reproducing all the pathological lesions in mice that are seen in cases of DHF, e.g. increase in vascular permeability, cerebral oedema, and alterations in leucocyte number and functions [2,3,18]. Mouse and hCF have shown a number of similarities, namely (i) hCF could be detected in ELISA, dot blot and Western blot by anti-mouse CF antisera; (ii) the electrophoretic migration of both the CF is the same, as shown in the Western blot test (Fig. 4); (iii) mRNA for hCF could be detected by oligonucleotide probes derived from the amino acid sequence of mouse CF; and (iv) cytotoxic activity on normal mouse spleen cells is similar by both of them. Efforts are being made to purify and determine the amino acid sequence of hCF so that its homology (if any) with mouse CF is determined. This will also explain the presence of cross-reacting antigens on the mouse CF and hCF. The cDNA library for mouse CF has been constructed and the recombinant CF has been expressed in *Escherichia coli* (U.C. Chaturvedi *et al.*, manuscript in preparation). There is a need to produce larger quantities of hCF so that it may be characterized fully.

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