

Identification, purification and characterization of a receptor for dengue virus-induced macrophage cytotoxin (CF2) from murine T cells

Pranay Deep Khare ^{*}, Meenakshi Khare ¹, Ritu Tandon, Umesh Chandra Chaturvedi ²

Postgraduate Department of Microbiology, K.G. Medical College, Lucknow 226-003, India

Received 24 April 2002; received in revised form 24 January 2003; accepted 9 March 2003

First published online 17 April 2003

Abstract

Dengue type-2 virus infection in mice induces a subpopulation of T lymphocytes to produce a cytokine cytotoxic factor, which induces macrophages (M ϕ) to produce a biologically active cytotoxic cytokine, the M ϕ cytotoxin (CF2). Previously we have identified the presence of intermediate-affinity receptors for CF2 on mouse peritoneal M ϕ . The present study was undertaken to identify the CF2-receptors (CF2-R) on murine T cells followed by their purification and characterization. Receptor binding assay and Scatchard analysis revealed single, high-affinity (1.0309 nM) receptors for CF2 on T cells (22 000 receptors per cell). The binding of [¹²⁵I]CF2 on murine T cells was saturable and specific. Furthermore, CF2-R was purified from normal mouse T cell plasma membrane by affinity chromatography followed by reversed-phase high-pressure liquid chromatography. The presence of CF2-R was confirmed by indirect dot-blot assay and its binding with [¹²⁵I]CF2. The purified CF2-R is a 90–95-kDa protein as characterized by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblot analysis. The chemical crosslinking of [¹²⁵I]CF2 and its receptor complex showed a product of 100–110 kDa on different subpopulations of murine T cells. The pretreatment of target cells with anti-CF2-R antisera inhibited the cytotoxic activity of CF2 in a dose-dependent manner and thus confirmed the biological significance of CF2-R. Moreover, the presence of CF2-R was also identified on normal human peripheral blood mononuclear cells and T and B cells by crosslinking with [¹²⁵I]CF2, thus revealing the possible role of CF2 and CF2-R in the immunopathogenesis of dengue virus disease.

© 2003 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Macrophage cytotoxin; Dengue virus; Receptor; Specific binding

1. Introduction

Dengue virus type-2 (DV-2) infection in mice produces immunosuppression characterized by spleen atrophy, reduced numbers of T lymphocytes, depressed humoral

and cell-mediated immune responses to heterologous and homologous antigens, and depressed macrophage (M ϕ) functions [1–3]. These features are caused by the development of an antigen specific suppressor pathway and a non-specific cytotoxic pathway in the spleen of DV-2-infected mice. Antigen specific immunosuppression is induced by the DV-2 specific suppressor cascade consisting of three generations of suppressor T cells and their soluble products (reviewed in [4]). Antigen non-specific immunosuppression observed in DV-2-infected mice is mediated by a mouse cytotoxic factor (mCF) produced by splenic T lymphocytes (Thy 1.2+, Lyt 1+23–) [1,2]. On native polyacrylamide gel electrophoresis (PAGE), mCF is a highly potent protein molecule of 45 kDa. It dissociates into two equally active heterodimer chains of 20 and 25 kDa on sodium dodecyl sulfate (SDS)–PAGE and has an isoelectric point of 6.5 [5]. The 19-amino acid

^{*} Corresponding author. Present address: Molecular Medicine Program, Mayo Clinic, Guggenheim 1842A, 200 First Street SW, Rochester, MN 55905, USA. Tel.: +1 (507) 266 4187; Fax: +1 (507) 284 8388.

E-mail address: khare.pranay@mayo.edu (P.D. Khare).

¹ Present address: Department of Immunology, Mayo Clinic, Rochester, MN 55905, USA.

² Present address: Industrial Toxicology Research Center, M.G. Road, Lucknow 226001, India.

N-terminal sequence of mCF has no homology to other known cytokines, T cell proteases, and other cytotoxic proteins [5]. The mouse cytotoxic factor kills mainly H-2A (a mouse major histocompatibility complex) negative M ϕ , T lymphocytes, and lymphoid cells of several species [2,3].

DV-2 specific mCF induces H-2A positive splenic and peritoneal M ϕ to produce another cytotoxin, CF2 [3]. CF2 is also produced in vivo in DV-2-infected mice as well as mice inoculated with mCF [6]. CF2 enhances the cytotoxic effect of mCF, and mCF and CF2 cause antigen non-specific immunosuppression in mice [3,5]. CF2 is a heat- and pH-labile, biologically active, cytotoxic protein with a molecular mass of 10–12 kDa. The first 10-amino acid sequence of CF2 from the C-terminus has no homology to the other known cytotoxic proteins and cytokines (unpublished data). CF2 kills H-2A negative and positive M ϕ , CD4⁺ and CD8⁺ T cells, and mast cells in 1 h by inducing Ca²⁺ influx into the target cells [7]. Cytotoxicity of CF2 is dependent on the Ca²⁺ and production of nitric oxide and superoxide ions in vitro and in vivo [7–9]. Also mCF and CF2 both produce various other immunopathological lesions in mice including increased capillary permeability and damage to the blood–brain barrier by release of histamine [10,11]. In addition, in vitro treatment of human blood leukocytes with mCF/CF2 decreases the E-rosetting capacity of T cells and alters the functions of monocytes [12,13]. Human dengue hemorrhagic fever/shock syndrome (DHF) is characterized by increased vascular permeability, thrombocytopenia, alterations in blood leukocytes, and cerebral edema [14]. It would thus appear that mCF and CF2 are both capable of reproducing the pathological lesions of DHF in mice, and mCF and CF2 are both pathogenesis-related proteins, at least in mouse dengue infection.

To further understand the mechanism of CF2 biology, we extended our studies to identify its cellular receptor on target cells. Cytokines act on cells in an autocrine and paracrine fashion through their receptor binding sites; CF2 kills CD4⁺ and CD8⁺ T cells and H-2A negative M ϕ in paracrine fashion and kills H-2A positive M ϕ in autocrine fashion [12]. CF2 gets adsorbed onto the plasma membrane of the target cells, and the cytotoxic activity of CF2 is inhibited by plasma membrane-stabilizing drugs [6,12]. The putative receptor binding sites for CF2 are neuraminidase-sensitive, suggesting that sialic acid residues are crucial for the formation of a receptor site for CF2 [12]. We have previously identified the presence of intermediate-affinity type of receptors on mouse M ϕ , and the binding of [¹²⁵I]CF2 to M ϕ was saturable and reversible, and dependent on temperature, pH and time [15]. The present study was undertaken to investigate the presence of a CF2-receptor (CF2-R) on mouse T cells, to purify and characterize it, and to identify the biologically active role of CF2-R in CF2-induced cytotoxicity.

2. Materials and methods

2.1. Animals

Inbred Swiss albino mice, aged 3–4 months, were obtained from the colony maintained in our department and used in experiments according to the guidelines of the animal facility of the institute.

2.2. Virus

DV-2 strain P23085 was obtained from the National Institute of Virology, Pune, India, and had undergone brain-to-brain passages in young mice. The virus strain produced 100% mortality after intracerebral (i.c.) inoculation in infant and adult mice. The infected mouse brain was ground in a chilled mortar and pestle with 3.6 ml of chilled Hank's balanced salt solution. The ground suspension was centrifuged at 3000 × g for 10 min at 4°C, and the supernatant was aliquoted and stored at –70°C for further use. The virus was used in doses of 1000 LD₅₀ in all experiments [16]. The virus titers were determined by i.c. inoculation in adult mice and LD₅₀ calculation by the method of Reed and Muench [17]. Normal mouse brain suspension was prepared similarly for control groups.

2.3. Cell preparations

Five ml of heparinized Eagle's minimum essential medium (MEM) containing 10% fetal calf serum (FCS) was inoculated by intraperitoneal (i.p.) injection into adult mice, and peritoneal lavages were aspirated into a 9-cm glass Petri dish. After incubation at 37°C for 2 h in the presence of 5% CO₂, the viability of glass adherent cells was assayed by exclusion of trypan blue dye. The purity of M ϕ was screened by their phagocytic activity of neutral red dye as described previously [13] and was 93–96% in different preparations. For T and B cells, a single cell suspension of the spleen cells was prepared in a 9-cm glass Petri dish. The glass non-adherent cells were poured into a MEM-treated nylon wool column for 1 h at 37°C to obtain enriched T and B cell populations [18,19]. The viability of cells was calculated using trypan blue dye, and the purity of cell preparations was assayed as described previously [20] and was 93–95% in different preparations. CD4⁺ and CD8⁺ cells were obtained by treatment of enriched T cells with CD8 and CD4 monoclonal antibody (Dako, Glostrup, Denmark), respectively. Human peripheral blood mononuclear cells and T and B cells were obtained as previously described [20].

2.4. Preparation and purification of CF

The CF was prepared from the spleens of DV-2-infected mice and purified by low-pressure liquid chromatography using a Sephacryl S-200 column as described earlier [5].

2.5. Preparation and purification of CF2

CF2 was prepared by the technique described previously [3]. Briefly, normal mouse M ϕ were treated with 5 μ g of CF at 4°C for 1 h in the presence of 5% CO₂. The cell sheet was washed and further incubated at 37°C for 24 h in the presence of normal saline. The adherent cells were scrapped off, sonicated, centrifuged at 3000 \times g, and the collected supernatant was assayed for cytotoxic activity [3,6]. The crude CF2 so prepared was purified with high-pressure liquid chromatography (HPLC; LKB-Pharmacia, Uppsala, Sweden) [11].

2.6. Cytotoxicity assay for CF/CF2

Normal mouse spleen cells were used as target cells to assay cytotoxic activity of CF/CF2 [1,3]. Briefly, the target cells (1×10^6) and the test solution (CF or CF2) were mixed in equal volume in a 96-well U-well perspex plate (Nunc, Denmark) and incubated at 4°C for 1 h. Viable nucleated cells were counted using trypan blue dye, and the percentage of non-viable cells was calculated. The data were corrected by deducting background non-viable cells.

2.7. Radiolabeling of CF2

CF2 was radiolabeled with ¹²⁵I (Bhabha Atomic Research Center, Mumbai, India) by the lactoperoxidase method [21] with some modifications. CF2 (500 μ g) was added to 0.5 mCi ¹²⁵I in the presence of 2 μ g of lactoperoxidase. The reaction was accelerated by addition of 0.88 mM H₂O₂ in four aliquots of 5 μ l each and terminated by addition of 25 μ l of lactoperoxidase stop buffer [phosphate-buffered saline (PBS), pH 7.0, saturated with tyrosine (10 mg ml⁻¹), 10% glycerol, 0.1% xylene cyanol]. The free iodine was separated from [¹²⁵I]CF2 by using a Sephacryl S-200 column as previously described [15].

2.8. Binding assay and Scatchard analysis

Purified mouse splenic T cells (1×10^6) were suspended in 1 ml MEM containing 10% FCS (pH 7.2). Various concentrations of [¹²⁵I]CF2 (5 pM to 15 nM) were added to the cells in the presence or absence of a 100-fold excess of unlabeled CF2 to assess specific binding, with a final inoculation volume of 1 ml in cryotubes (Nunc, Denmark). Radioactivity of the cells was counted in a gamma counter (LKB-Pharmacia). Non-specific binding, determined in the presence of a 100-fold excess of unlabeled CF2, was subtracted from the total binding to give specific binding. Data from the binding experiments were subjected to Scatchard analysis [22]. For inhibition studies, [¹²⁵I]CF2 (12 nM) was preincubated with anti-CF2 antisera or heterologous DV-2 specific anti-suppressor factor (SF) antisera for 1 h at 37°C prior to binding.

2.9. Preparation of purified plasma membrane from T lymphocytes

Membrane preparation was performed as described previously [23–25] with some modifications. The normal mouse T cells ($6\text{--}8\times 10^8$ ml⁻¹) were purified as described above and washed twice with PBS and sedimented at 600 \times g for 10 min. The pellet was dissolved with an equal volume of PBS supplemented with 2 mM phenyl methyl sulfonyl fluoride, 1 mM pepstatin, 1 mM leupeptin and 2 mM *O*-phenanthroline. The cells were lysed by sonication, and the disrupted cell suspension was centrifuged at 2000 \times g for 10 min. The supernatant was centrifuged at 20 000 \times g for 1.5 h to obtain the crude plasma membrane pellet, which was then dissolved in PBS containing protease inhibitors and 1% Triton X-100. The solution was magnetic-stirred for 30 min at 4°C and then centrifuged at 105 000 \times g for 1.5 h (Sorvall S-28, Asheville, NC, USA). The resultant supernatant was collected and used as purified T cell plasma membrane.

2.10. Purification of CF2-R by affinity chromatography

HPLC-purified CF2 (20 μ g) was dissolved in the coupling buffer (0.1 M sodium bicarbonate, 0.5 M NaCl, pH 8.3) and coupled to cyanogen bromide (CNBr)-activated Sepharose 4B according to the manufacturer's suggestions. Approximately 80% of the CF2 were coupled to the gel, and after washing, the purified T cell plasma membrane preparation was adsorbed onto the CF2-coupled CNBr-activated beads for 1 h at 37°C. The column was washed with 50 mM Tris-HCl (pH 8.0) to remove the unbound membrane protein. The receptor was then eluted with 0.2 M glycine-HCl (pH 2.5), and the pH was adjusted with 0.2 M Tris-HCl (pH 9.2) to pH 7.2. Protein concentrations of eluted fractions were determined using bovine serum albumin (BSA) as a standard [26].

2.11. Dot-blot assay

The nitrocellulose dot-blot assay was performed to identify the presence of CF2-R protein purified from the CF2-affinity column. Different fractions of receptor protein were applied to the nitrocellulose membrane in a dot-blot apparatus (Millipore, Bedford, MA, USA). The membrane was blocked with 3% BSA in 10 mM Tris-HCl (pH 7.4) overnight. The blots were washed with PBS-T (Tween-20, 0.05%) and treated with 5 μ g of CF2 for 1 h at 37°C. The blots were washed and incubated with anti-CF2 antisera at room temperature followed by horseradish peroxidase conjugate anti-mouse IgG (Sigma, St. Louis, MO, USA) and developed with a substrate containing diaminobenzidine (Sigma) and hydrogen peroxide (Sigma).

2.12. Purification of CF2-R by HPLC

The affinity-purified CF2-R was further purified by a Shoedex protein column (KW-803, Millipore) using reversed-phase (RP)-HPLC. The column was preequilibrated with 0.5 M Tris-HCl (pH 7.4). The different fractions of receptor protein were first dissolved in 0.5 M Tris-HCl (pH 7.4), and 100 μ l of sample was loaded into the injection loop. The protein was eluted with the same solvent, and chromatographic elution was monitored by absorbance at 280 nm. In some experiments, the CF2-R was treated with 5 mM of dithiothreitol (DTT, Sigma). The purified protein peak was collected in Eppendorf tubes, and the protein content was estimated.

2.13. Preparation of antisera

Antisera against CF2-R were raised in mice by injecting 5 μ g of purified CF2-R emulsified in Freund's complete adjuvant (Sigma) i.p. at day 0, followed by a booster dose of 5 μ g CF2-R in Freund's incomplete adjuvant (Sigma) i.p. 15 days later. Blood was collected on day 30, and the separated antisera were pooled and stored at -20°C . A similar protocol was used to prepare anti-CF2 antisera and anti-SF and anti-SF-receptor (SF-R) antisera [27].

2.14. SDS-PAGE and immunoblotting

The HPLC-purified CF2-R was run on a 12% SDS-PAGE gel along with molecular mass markers according to the method of Laemmli [28]. Some gels were silver stained, and some were electroblotted onto nitrocellulose membrane, treated with anti-CF2-R antisera, and developed as described above.

2.15. Amino acid sequence analysis of CF2-R by mass spectroscopy (MS)

The electrophoretic bands stained with Coomassie Blue were excised from the gel with a scalpel and cut into pieces no larger than 2 mm². Prior to digestion, the gel pieces were destained with 50% acetonitrile/50 mM Tris, pH 8.1, until clear, and then reduced and alkylated with 20 mM DTT and 40 mM iodoacetamide. In situ enzymatic digestion was performed overnight at 37°C with modified sequencing grade trypsin (Promega Corporation, Madison, WI, USA), using 0.01 μ g μ l⁻¹ in 20 mM Tris, pH 8.1. The peptides were extracted from the gel and separated with an 140D capillary HPLC system (Applied Biosystems, Foster City, CA, USA) running a 300 μ m \times 100 mm Vydac C18 MS 5- μ m column with an acetonitrile gradient in the presence of 0.1% formic acid and a flow of 4 μ l min⁻¹. The flow was directed into a LCQ Deca ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA). The ion trap was set to run in data-dependent triple play mode consisting of full scan (400–1900 amu), zoom scan, fol-

lowed by MS/MS mode. The MS/MS data were correlated using the SEQUEST search algorithm with tryptic peptide sequences from the National Center for Biotechnology Information (NCBI) database and Basic Local Alignment Search Tool (BLAST) protein search.

2.16. Chemical crosslinking

For affinity crosslinking, the target cells (2×10^6) were incubated with [¹²⁵I]CF2 (15 nM) for 1 h at 37°C in the presence or absence of 100-fold excess of unlabeled CF2. After washing, the cells were suspended in PBS containing 10 mM MgCl₂ (pH 8.4), and 25 μ l of crosslinker disuccinimidyl suberate (10 mg ml⁻¹ suspended in dimethyl sulfoxide, Sigma) for 30 min at 4°C. The reaction was quenched by using 2.5 ml cold Tris-HCl (pH 7.4) and 1 mM EDTA. The cell suspension was centrifuged, and the cell pellet was lysed with 50 μ l of lysis buffer containing 2 mM phenyl methyl sulfonyl fluoride, 126 mM Tris-HCl (pH 7.5), 0.1% Triton X-100 and 10 mM EDTA for 30 min at 4°C. Lysed cells were centrifuged at 10 000 \times g at 4°C, and the supernatant was fractionated on 12% SDS-PAGE. The gels were dried using a gel drier (LKB Pharmacia) and exposed to Konika AX 2355 film (Konica Co., Mahwah, NJ, USA). The films were developed after exposure of 10–15 days at -70°C . For detection of CF2-R in affinity-purified proteins, the different fractions of eluted proteins were applied to the nitrocellulose membrane in a dot-blot apparatus followed by treatment with [¹²⁵I]CF2 (12 nM) for 1 h at 37°C. The membrane was then washed, and films were exposed and developed.

3. Results

3.1. Saturation of binding on normal T cells

The saturation of binding of [¹²⁵I]CF2 to normal mouse T cells was examined (Fig. 1A). A fixed number of cells (1×10^6) were incubated with an increasing concentration of [¹²⁵I]CF2 (5 pM to 15 nM) in the presence or absence of 100-fold excess of unlabeled CF2. Specific binding was calculated by subtracting the binding in excess of unlabeled CF2 (non-specific binding) from the total binding (binding in the absence of unlabeled CF2). The specific binding was increased in a dose-dependent manner and was saturated with 12.0 nM [¹²⁵I]CF2.

3.2. Scatchard plot analysis of binding of [¹²⁵I]CF2 to normal T cells

The data obtained from the specific binding curve were subjected to Scatchard analysis [22]. The results showed only one regression line, suggesting the presence of high-affinity receptors (Fig. 1B). The negative inverse of the regression coefficient gave a dissociation constant (K_d) of

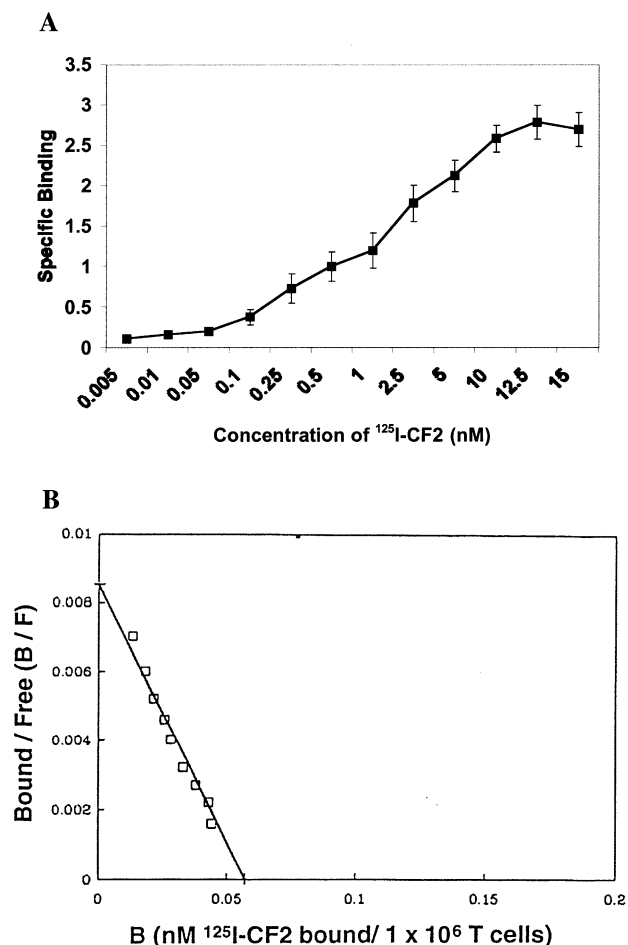


Fig. 1. Specific binding of CF2 to normal mouse T cells. A: Normal mouse T cells (1×10^6) were incubated with increasing concentration of [^{125}I]CF2 in the presence or absence of a 100-fold excess of unlabeled CF2. Specific binding was calculated by subtraction of non-specific binding (binding in the presence of unlabeled CF2) from total binding (in the absence of unlabeled CF2). The results are expressed as the amount of specifically bound [^{125}I]CF2 (nM)/ 10^6 cells. All experiments were performed in triplicate. B: Equilibrium binding of [^{125}I]CF2 to T cells was analyzed by the Scatchard method. Each point was calculated by determining the moles of specifically bound (B) and free (F) CF2 and plotting them as B/F versus B . The line of best fit was constructed according to the negative inverse of the regression coefficient.

1.0309 nM, and the number of receptor sites was 22 000 per T cell.

3.3. Anti-CF2 antisera inhibits the binding of [^{125}I]CF2 to normal T cells

The specificity of the binding of the [^{125}I]CF2 to normal

mouse T cells was investigated using anti-CF2 antisera. The [^{125}I]CF2 was incubated with either anti-CF2 antisera or anti-SF antisera as control prior to binding. Anti-CF2 antisera blocked the binding of [^{125}I]CF2 to normal T cells (80% inhibition), while anti-SF antisera had no significant effect (5% inhibition) (Table 1).

3.4. Purification and detection of CF2-R by immunoaffinity column

Several individual lots of CF2-R were purified from the equivalent of 20 mice splenic T cell plasma membranes as described above. The screening of binding of CF2-R with CF2 was examined by dot-blot assay at each level of the plasma membrane purification steps (data not shown). The purified plasma membrane protein (1260 μg) obtained by the above method was adsorbed onto a CF2-adsorbed CNBr-activated Sepharose 4B column. The non-specific adsorbed protein was first eluted until the absorbance at 280 nm of the output solution was at the baseline, then CF2-R was eluted from the column with glycine-HCl buffer (data not shown). Each fraction was collected, and the protein concentration of each fraction was detected. About 15 μg of the total CF2-R was obtained from the affinity-purification method. Screening for CF2-R was done by directly applying each fraction obtained from above method onto the nitrocellulose membrane and detecting with anti-CF2 antisera. Fractions eluted with glycine-HCl buffer showed the maximum binding, suggesting the presence of a specific receptor for CF2 (data not shown). No reaction was observed with normal mouse sera or heterologous anti-SF antisera. The fractions eluted with Tris buffer showed no reactivity with anti-CF2 antisera (data not shown) and thus confirmed the presence of CF2-R in the eluate of glycine-HCl buffer. To further confirm the presence of CF2-R, each affinity-purified fraction was blotted onto nitrocellulose membrane and treated with [^{125}I]CF2 and only glycine-HCl-eluted fractions showed the specific binding to [^{125}I]CF2 and, hence, determined the presence of CF2-R in these fractions (data not shown).

3.5. Purification and characterization of CF2-R

Each fraction containing CF2-R was collected and run on the Shoedex protein column of RP-HPLC using 0.5 M Tris-HCl (pH 7.4) as the solvent. The proteins were monitored at the 280 nm absorbance range as described above.

Table 1

	No antibody	Anti-CF2 antibody	Anti-SF antibody
Specific binding ^a (cpm/ 1×10^6 T cells)	2700 (± 245)	520 (± 45)	2580 (± 260)
% Inhibition	0	80	5

^a[^{125}I]CF2 was incubated with anti-CF-2 antibody or with anti-SF antibody prior to binding. Specific binding was calculated by incubating T cells (1×10^6) with 12 nM [^{125}I]CF2 in the presence or absence of a 100-fold excess of unlabeled CF2. Data represent the mean values of three experiments.

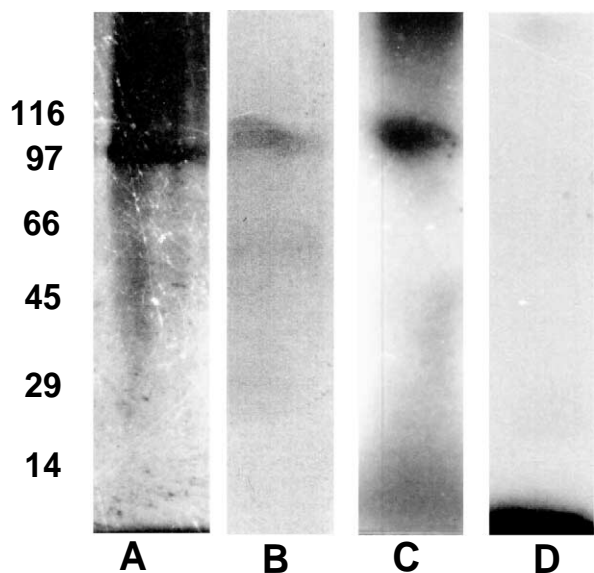


Fig. 2. Characterization of purified CF2-R. A: Silver staining of CF2-R on 12% SDS-PAGE; B: Western immunoblot of CF2-R; CF2-R reaction with anti-CF2-R antisera was visualized with a substrate. Chemical crosslinking of [125 I]CF2 to CF2-R on T cells in the absence (C) or presence (D) of 100-fold excess unlabeled CF2 as described above. The left panel shows the molecular mass markers of standard proteins.

The elution profile of CF2-R resolved into a single peak and further treatment of purified CF2-R with DTT also showed a single peak indicating that the CF2-R consists of a single chain (data not shown). The protein collected from the RP-HPLC was run on a 12% SDS-PAGE gel along with molecular mass markers of standard protein, yielding a single band corresponding to 90–95 kDa (Fig. 2A). The specificity of the protein was further confirmed by immunoblotting. Fig. 2B indicates that anti-CF2-R antisera reacted specifically to the CF2-R and resolved into a single band. No reaction was observed with normal mouse sera and anti-SF-R antisera (data not shown).

3.6. Amino acid sequence analysis of CF2-R

The purified CF2-R was excised from a 12% SDS-PAGE gel and proteins were digested with trypsin for overnight. The peptides were separated by capillary HPLC and run on MS for their sequence analysis as described above. The data were correlated using the SEQUEST from the NCBI database and NCBI-BLAST

Table 2

Amino acid sequence of CF2-R^a

Peptide 1	I E I S E L N R
Peptide 2	L D S E L K
Peptide 3	L N D L E D A L Q Q A K
Peptide 4	L S L N N Q F A S F I D K
Peptide 5	T L L E G E E S R
Peptide 6	Y E E L Q I T A G R

^aCF2-R was excised from SDS-PAGE gel and proteins were digested with trypsin. The peptides were separated by HPLC and run on MS for sequence analysis as described in Section 2. Amino acids are shown by single letters.

protein search. The partial amino acid sequence of CF2-R was determined (Table 2). The partial amino acid sequence analysis of CF2-R did not match with the known cytokine, cytokine receptor and other known cytotoxic proteins. It was observed that the sequence is hydrophilic in nature with serine (S), threonine (T), lysine (K) and arginine (R) on each peptide.

3.7. Chemical crosslinking of CF2 on mouse and human cells

Crosslinking experiments were performed for the structural analysis of the CF2-R on different mouse or human cells. A major crosslinked product of 100–105 kDa was seen with normal mouse T cells (Fig. 2C). The finding indicated that CF2-R on normal mouse T cells has a molecular mass of 90–95 kDa (after subtracting 10 kDa of CF2). Crosslinking of radiolabeled CF2 to normal mouse T cells was completely inhibited if the cells were preincubated in 100-fold excess of unlabeled CF2 (Fig. 2D). Crosslinking was also performed on the normal mouse M ϕ and B cells and showed one crosslinked product, while normal mouse CD4⁺ and CD8⁺ cells showed two crosslinked products (Table 3). The normal human peripheral blood mononuclear cells and T and B cells were also examined by crosslinking with [125 I]CF2 and showed one crosslinked product of about the same size as on mouse T cells (Table 3).

3.8. Effect of anti-CF2-R antisera on CF2-induced cytotoxicity on mouse cells

This experiment was designed to see whether CF2 acts through its receptor. The target cells were treated with

Table 3

Comparative structural analysis of CF2-R on different mouse and human cells obtained from chemical crosslinking

	Mouse					Human		
	T cells	CD4 ⁺	CD8 ⁺	B cells	M ϕ	T cells	B cells	PBMC ^a
Crosslinked product (kDa)	100–105	30–35 100–110	105–110 125–130	100–105	100–105	90–105	90–105	90–105

[125 I]CF2 was added to different target cells and crosslinked with disuccinimidyl suberate. Cells were lysed, and analyzed by 12% SDS-PAGE followed by autoradiography. Molecular masses of crosslinked products were determined.

^aPBMC: peripheral blood mononuclear cells.

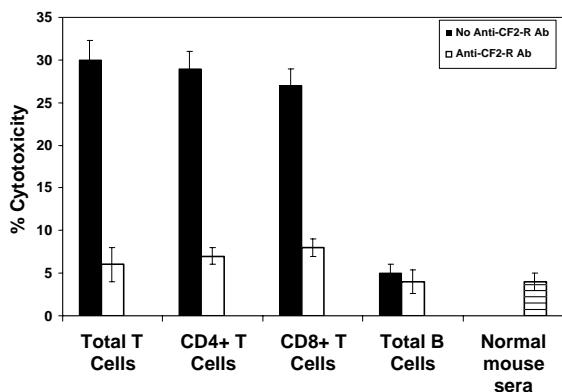


Fig. 3. Effect of anti-CF2-R antisera on CF2-induced cytotoxicity. Target cells (1×10^6) were pretreated with anti-CF2-R antisera followed by treatment with CF2 as described above. The bars represent the standard deviation of three individual experiments.

anti-CF2-R antisera (1:100 dilution) for 1 h at 37°C prior to treatment with CF2. The data presented in Fig. 3 showed 70–75% inhibition of cytotoxic activity of CF2 on CD4⁺ and CD8⁺ cells and total T cells when the cells were pretreated with anti-CF2-R antisera. Because CF2 cannot kill normal mouse B cells, there is no inhibition of cytotoxicity on these cells. This suggests that CF2 exerts its biological effects by binding to its receptor on the cell surface.

4. Discussion

The findings in the present study extend our previous report on the identification of a receptor for DV-2-induced CF2 [15]. The results reported herein establish the possibility of the presence of a specific receptor for DV-2-induced CF2 on different cells. [¹²⁵I]CF2 bound to CF2-R present on T cells with high affinity with a K_d of 1.0309 nM, and the number of receptor sites was 22 000 per T cell. The binding of [¹²⁵I]CF2 to CF2-R present on normal T cells is blocked (80% inhibition) only by anti-CF2 antisera, while irrelevant anti-SF antisera have no significant effect (5% inhibition) on the binding (Table 1). In our previous studies we have also identified the presence of CF2-R on mouse peritoneal M ϕ , and the CF2-R was saturable with 15 nM [¹²⁵I]CF2. Scatchard analysis revealed the presence of intermediate-affinity receptors for CF2 on peritoneal M ϕ with a dissociation constant of 14.28 nM and 1.1×10^6 receptor sites per cell [15]. Scatchard analysis indicated that interaction of CF2 with its receptor fits with a bimolecular association of the peptide and a population of non-interacting receptor sites. This is consistent with dissociation experiments, which indicate simple first order kinetics. Cytokines bind to their receptors with high, low, and intermediate affinities. The receptor for the tumor necrosis factor (TNF) [29], granulocyte-M ϕ colony-stimulating factor (GM-CSF) [30] and M-CSF are of only high affinity [31]. There are high- and low-affinity receptors for

interleukin-5 (IL-5) [31], IL-7 [32], and DV-2-induced SF [27]. IL-2 has all three – high-, low-, and intermediate-affinity receptors [23]. This study suggests the presence of high-affinity receptors on murine T cells. Therefore, T cells were preferred over peritoneal M ϕ for the purification of CF2-R.

Solubilization of T cell plasma membrane was performed in the presence of 1% Triton X-100 to replace its hydrophobic interaction with detergent molecules and to solubilize the receptor protein from the plasma membrane in the presence of protease inhibitors. Affinity purification of receptor proteins usually involves (i) immobilization of the ligand, (ii) adsorption of a crude, solubilize sample containing the receptor protein of interest, (iii) elution of non-specifically adsorbed material by high salt or change in pH, and (iv) specific elution of the receptor protein using a low-pH buffer. In the present study, CF2 was immobilized with CNBr-4B beads in a column, and the purified T cell plasma membrane was adsorbed on it. Receptor isolated from affinity chromatography shows several 100-fold purity to homogeneity and high binding specificity to its ligand as observed by the above purification methods. Inhibitors for TNF- α and TNF- β were also purified to homogeneity by a combination of affinity chromatography, HPLC, and SDS-PAGE [24]. The receptors for various cytokines, such as G-CSF [31] and GM-CSF [30] have been purified by the above methods and each is composed of single bands. RP-HPLC achieved purification of CF2-R in a single sharp peak, and SDS-PAGE determined the CF2-R molecular mass corresponding to 90–95 kDa. IL-2R eluted by RP-HPLC gave a single peak and a single band [23], DV-2-induced SF-R split into two peaks by RP-HPLC [27], and IL-1 receptor antagonist (IL-1Ra) gave three species of native IL-1Ra by RP-HPLC [33].

With the above analysis, it was difficult to distinguish between physiological and artifactual binding and whether one or more proteins are associated with an individual receptor. To resolve such ambiguities, chemical crosslinking was studied by homobifunctional crosslinking reagent disuccinimidyl suberate, which covalently couples adjacent amino groups. This reagent has been used extensively to identify the cellular receptors for a number of cytokines such as TNF [29], T cell growth factor [34], and IL-5 [35]. The finding presented here showed the presence of a 100–105-kDa crosslinked product, and the binding was completely inhibited with preincubation of 100-fold excess of unlabeled CF2. These data support the implication that CF2-R could be the receptor for CF2. The binding of CF2 to CF2-R was specific and is supported as follows: (i) by finding a marked inhibition of binding by pretreatment with anti-CF2 antisera but not by heterologous antisera; (ii) by finding that purified CF2-R (affinity and RP-HPLC) reacted only with anti-CF2-R antisera but not with heterologous (anti-SF and anti-SF-R) antisera or normal mouse antisera; (iii) by crosslinking of [¹²⁵I]CF2

on murine T cells, and finding that this specific binding was significantly inhibited by excess of unlabeled CF2 (Fig. 2C,D); and (iv) by finding that pretreatment of target cells with anti-CF2-R antisera significantly inhibited CF2-induced cytotoxicity (Fig. 3). This study also suggests the active role of CF2-R in CF2-induced cell death mechanism at least in vitro, and its possible role in vivo. These findings were similar to that for anti-TNF- α antibodies, which inhibited the cytotoxic effect of TNF- α [29]. The partial amino acid sequences of CF and CF2 have no homology to other known cytokines. The analysis of the partial amino acid sequence of CF2-R suggests that CF2-R has no homology with other known cytokine, cytokine receptor and cytotoxic protein families, which suggests that CF2-R could be a novel receptor protein.

The previous study revealed that CF2-induced DHF-like symptoms in mice [10,11], and recent progress in cytokine research has provided new insight into the pathogenesis of DHF [36–38]. The high levels of soluble IL-2R, soluble CD4 and CD8 [37], and IL-1Ra [38] were detected in DHF patients, indicating a progressive and important role of cytokines and their receptors in the immunopathogenesis of DHF. The characterization of the CF2-R is of interest not only because it elucidates a new aspect of CF2 biology, but also because it may broaden the understanding of CF2-induced immunopathological lesions in mice. The presence of CF2-R on normal human cells may suggest a new role of CF2 and CF2-R in the pathogenesis of DHF and may provide an important approach in the treatment and prevention of DHF. The DHF-like pathological lesions produced by mCF in mice can be prevented by active vaccination of mice using mCF as an antigen. The immunized mice were protected, which resulted in the absence of clinical symptoms of the disease against a subsequent challenge with mCF as well as with a lethal i.c. dose of DV-2 [39]. These studies suggest a vaccine strategy that should be directed against the primary cause of the disease (the cytokine) rather than the infective agent [39]. The present study also indicates the need to look for expression of CF2 and its possible receptor CF2-R in such cases.

Acknowledgements

The authors are thankful to Dr. D.N. Rao, Additional Professor, Biochemistry Department, All India Institute of Medical Sciences, New Delhi, India, for providing the HPLC facility. The authors also thank Dr. M.J. Federspiel and B.J. Madden, Mayo Clinic, Rochester, MN, USA, for their support and help in protein sequencing. Troy Wegman, Mayo Clinic Rochester is acknowledged for the critical reading and editing of the manuscript. This work and P.D.K. were supported by financial assistance from Lady Tata Memorial Trust, Mumbai, India, and Council for Scientific and Industrial Research (CSIR), N. Delhi, India.

References

- [1] Chaturvedi, U.C., Bhargava, A. and Mathur, A. (1980) Production of cytotoxic factor in the spleens of dengue virus infected mice. *Immunology* 40, 665–671.
- [2] Chaturvedi, U.C. (1986) Virus-induced cytotoxic factor in AIDS and Dengue. *Immunol. Today* 7, 159.
- [3] Gulati, L., Chaturvedi, U.C. and Mathur, A. (1983) Dengue virus-induced cytotoxic factor induce macrophage to produce cytotoxin. *Immunology* 49, 121–130.
- [4] Chaturvedi, U.C. (1984) Dengue virus induced suppressor pathway. *Curr. Sci.* 53, 971–976.
- [5] Khanna, M. and Chaturvedi, U.C. (1992) Purification and amino-terminal sequence of the dengue virus-induced cytotoxic factor. *Int. J. Exp. Pathol.* 73, 43–49.
- [6] Gulati, L., Chaturvedi, U.C. and Mathur, A. (1986) Production of dengue virus-induced macrophage cytotoxin in vivo. *Br. J. Exp. Pathol.* 67, 269–277.
- [7] Dhawan, R., Chaturvedi, U.C., Khanna, M., Mathur, A., Tekwani, B.L. and Pandey, V.C. (1991) Obligatory role of Ca²⁺ in the cytotoxicity of dengue virus-induced cytotoxin. *Int. J. Exp. Pathol.* 72, 31–39.
- [8] Mukerjee, R., Misra, A. and Chaturvedi, U.C. (1996) Dengue virus-induced cytotoxin releases nitrite by spleen cells. *Int. J. Exp. Pathol.* 77, 45–51.
- [9] Misra, A., Mukerjee, R. and Chaturvedi, U.C. (1996) Release of reactive oxygen intermediates by dengue virus-induced macrophage cytotoxin. *Int. J. Exp. Pathol.* 77, 237–242.
- [10] Dhawan, R., Khanna, M., Chaturvedi, U.C. and Mathur, A. (1990) Effects of dengue virus-induced cytotoxin on capillary permeability. *Int. J. Exp. Pathol.* 71, 83–88.
- [11] Dhawan, R., Khanna, M., Chaturvedi, U.C. and Mathur, A. (1994) Dengue virus-induced cytokine damages the blood brain barrier. *Proc. Ind. Natl. Sci. Acad.* B601, 45–52.
- [12] Gulati, L., Chaturvedi, U.C. and Mathur, A. (1984) Study of target cell receptor sites for dengue virus-induced cytotoxin. *Ind. J. Med. Microbiol.* 2, 235–242.
- [13] Chaturvedi, U.C., Gulati, L. and Mathur, A. (1982) Inhibition of E-rosette formation and phagocytosis by human blood leukocytes after treatment with the dengue virus-induced cytotoxic factor. *Immunology* 45, 679–685.
- [14] Bhamarapravati, N. (1993) Pathology of dengue haemorrhagic fever. In: *Monograph on Dengue/Dengue Haemorrhagic Fever* (Thongchroen, P., Ed.), pp. 72–79. WHO-SEARO, New Delhi.
- [15] Khare, P.D., Dhawan, R. and Chaturvedi, U.C. (1996) Identification and characterization of receptor for dengue virus induced macrophage cytotoxin. *Ind. J. Exp. Biol.* 34, 652–657.
- [16] Chaturvedi, U.C., Dhawan, R., Khanna, M. and Mathur, A. (1991) Breakdown of the blood-brain barrier during dengue virus infection of mice. *J. Gen. Virology* 72, 859–866.
- [17] Reed, L.J. and Muench, H.A. (1938) A simple method of estimating fifty percent endpoints. *Am. J. Hygiene* 27, 493–497.
- [18] Julius, M.H., Simpson, E. and Herzenberg, L.A. (1973) A rapid method for the isolation of functional thymus-derived murine lymphocytes. *Eur. J. Immunol.* 3, 645–649.
- [19] Trizio, D. and Cudkovic, G. (1974) Separation of T and B lymphocytes by nylon wool columns: evaluation of efficacy by functional assay in vivo. *J. Immunol.* 113, 1093–1097.
- [20] Agarwal, R., Chaturvedi, U.C., Misra, A., Mukerjee, R., Kapoor, S., Nagar, R., Tandon, R. and Mathur, A. (1998) Production of cytotoxic factor by peripheral blood mononuclear cells (PBMC) in patients with dengue hemorrhagic fever. *Clin. Exp. Immunol.* 112, 477–481.
- [21] Marchalonis, J.J. (1969) An enzymatic method for the trace iodination of immunoglobulins and other proteins. *Biochem. J.* 113, 299–305.

- [22] Scatchard, G. (1949) The attractions of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* 51, 660–672.
- [23] Urdal, D.L., March, C.J., Gillis, S., Larsen, A. and Dower, S.K. (1984) Purification and chemical characterization of the receptor for interleukin 2 from activated human T lymphocytes and from a human T-cell lymphoma cell line. *Proc. Natl. Acad. Sci. USA* 81, 6481–6485.
- [24] Gatanga, T., Hwang, C.D., Kohr, W., Cuppucini, F., Lucci III, J.A., Jeffes, E.W., Lentz, R., Tomich, J., Yamamoto, R.S. and Granger, G.A. (1990) Purification and characterization of an inhibitor (soluble tumor necrosis factor receptor) for tumor necrosis factor and lymphotoxin obtained from the serum ultrafiltrates of human cancer patients. *Proc. Natl. Acad. Sci. USA* 87, 8781–8784.
- [25] Gavin III, J.R., Buell, D.N. and Roth, J. (1972) Preparation of solubilized insulin receptors from human lymphocytes. *Biochem. Biophys. Res. Commun.* 49, 870–876.
- [26] Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193, 265–275.
- [27] Mukherjee, R., Chaturvedi, P. and Chaturvedi, U.C. (1993) Identification and purification of a receptor on macrophages for the dengue virus-induced suppressor cytokine. *Clin. Exp. Immunol.* 91, 257–265.
- [28] Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- [29] Kull Jr., F.C., Jacobs, S. and Cuatrecasas, P. (1985) Cellular receptor for ¹²⁵I-labelled tumor necrosis factor: specific binding, affinity labelling and relationship to sensitivity. *Proc. Natl. Acad. Sci. USA* 82, 5756–5760.
- [30] Baldwin, G.C., Golde, D.W., Widhopf, G.F., Economou, J. and Gassan, J.C. (1991) Identification and characterization of a low-affinity granulocyte-macrophage colony-stimulating factor receptor on primary and cultured human melanoma cells. *Blood* 78, 609–615.
- [31] Nicola, N.A. and Peterson, L. (1986) Identification of distinct receptor for two hemopoietic growth factor (granulocyte colony stimulating factor and multipotential colony-stimulating factor) by chemical cross linking. *J. Biol. Chem.* 261, 12384–12389.
- [32] Cosman, D., Lyman, S.D., Idzerda, R.L., Beckman, M.P., Pak, L.S., Goodwin, R.G. and March, C.J. (1990) A new cytokine receptor superfamily. *Trends Biochem. Sci.* 15, 265–270.
- [33] Arend, W.P. (1991) Interleukin 1 receptor antagonist. A new member of the interleukin 1 family. *J. Clin. Invest.* 88, 1445–1451.
- [34] Leonard, W.J., Depper, J.M., Robb, R.J., Waldman, T.A. and Greene, W.C. (1983) Characterization of human receptor for T-cell growth factor. *Proc. Natl. Acad. Sci. USA* 80, 6957–6961.
- [35] Ingle, E. and Young, I.G. (1991) Characterization of a receptor for interleukin-5 on human eosinophils and myeloid leukemia line HL-60. *Blood* 78, 339–344.
- [36] Chaturvedi, U.C., Agarwal, R., Elbishbishi, E.A. and Mustafa, A.S. (2000) Cytokine cascade in dengue haemorrhagic fever: implications for pathogenesis. *FEMS Immunol. Med. Microbiol.* 28, 183–188.
- [37] Kurane, I., Innis, B.L., Nimmannitya, S., Nisalak, A., Meager, S., Janus, J. and Ennis, F.A. (1991) Activation of T lymphocytes in dengue virus infection: High levels of soluble interleukin 2 receptor, soluble CD4, soluble CD8, interleukin 2 and interferon- γ in sera of children with dengue. *J. Clin. Invest.* 88, 1473–1480.
- [38] Chang, D.M. and Shaio, M.F. (1994) Production of interleukin-1 (IL-1) and IL-1 inhibitor by human monocytes exposed to dengue virus. *J. Infect. Dis.* 170, 811–817.
- [39] Chaturvedi, U.C., Mukerjee, R. and Dhawan, R. (1994) Active immunization by a dengue virus-induced cytokine. *Clin. Exp. Immunol.* 96, 202–207.