
An enzyme immunoassay for detection of Japanese encephalitis virus-induced chemotactic cytokine

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Japanese encephalitis virus (JEV) induces human peripheral blood monocytes to secrete a chemotactic cytokine [human macrophage-derived factor (hMDF)] which causes chemotaxis of neutrophils. The only known assay for hMDF cannot quantify its level in samples, so an enzyme immunoassay has been standardized for detection of hMDF and hMDF-specific antibodies in test samples. The reported enzyme linked immunosorbent assay (ELISA) was found to be sensitive (89%), specific (91%), accurate (92.2%) and reproducible and was able to detect a minimum concentration of 23 ng hMDF/ml in test samples. The chemotactic factor could be detected in JEV inoculated mouse sera and JEV infected culture fluids. Significant finding of the test was the detection of hMDF in sera of human cases of JE.

1. Introduction

Enzyme linked immunosorbent assay (ELISA) is a highly sensitive technique which is specific, less time consuming and reproducible method for detection and quantification of many cytokines (Beech *et al* 1997; Jung *et al* 1998). Widely used bioassays, which measure the chemotactic activity are not easily applied to clinical samples because these assays are not sensitive enough, time consuming and are easily affected by other factors (Ida *et al* 1992). Human cytokine measurements are now mostly performed with commercially available or home made ELISA procedures (Ledur *et al* 1995).

Japanese encephalitis virus (JEV, an arthropod-borne flavivirus) infection remains one of the major causes of encephalitis with significant mortality among children in south-east Asia, including India. The most frequent alteration during viral infections is leucocytopenia with lymphocytopenia (de Gruchy 1976); but JEV infection is associated with leucocytosis and neutrophilia (Chaturvedi *et al* 1979). An early influx of macrophages followed by neutrophils at the site of injury in different human organs (Johnson *et al*

1985) and in experimental animals (Mathur *et al* 1988) has been reported, yet the mechanism of recruitment of these cells is undefined. We have shown that JEV induces splenic macrophages in mice and peripheral blood monocytes in humans to secrete a highly potent chemotactic factor, peptide of low molecular weight (\sim 10 kDa), which causes chemotaxis of neutrophils and is named macrophage derived factor (MDF, Khanna *et al* 1991) and human macrophage-derived factor (hMDF, Singh *et al*, unpublished results) respectively. It is a biologically active protein (Mathur *et al* 1992; Khanna *et al* 1993, 1994; Srivastava *et al* 1999). The *in vivo* studies in mice have incriminated hMDF to play an important role in pathogenesis of JEV infection (Singh *et al*, unpublished results). It causes increase in capillary permeability and break down of blood-brain barrier, resulting in leakage of Evans blue dye bound protein into peritoneal cavity and into brain substance. The only method for detection of hMDF is the chemotactic activity assay, by which the level cannot be quantified. Since hMDF is a potent pathogenesis related protein, accurate determination of it in the samples may be of great

Keywords. Enzyme immunoassay; human macrophage-derived factor; Japanese encephalitis virus

Abbreviations used: ELISA, Enzyme linked immunosorbent assay; JEV, Japanese encephalitis virus; hMDF, human macrophage-derived factor; HBSS, Hank's balanced salt solution; CSF, cerebrospinal fluid; MEM, minimum essential medium; FCS, foetal calf serum; PBS, phosphate buffered saline; NS, normal saline; As, antisera; HRP, horse raddish peroxidase.

importance. Therefore, the present study was undertaken to develop an ELISA for detection of hMDF and hMDF-specific antibodies in clinical samples.

2. Materials and methods

2.1 Animals and virus

The study was performed on 4–8 weeks old inbred conventional Swiss albino mice, obtained from the mouse colony maintained in the Department of Microbiology, KG Medical College, Lucknow. JEV strain 78668A (Mathur *et al* 1982) was used in the form of infected adult mouse brain suspension throughout the study.

2.2 Human peripheral blood cell culture

Human heparinized (10 U heparin/ml) venous blood was obtained and allowed to stand at 37°C for 1 h for plasma to separate. Buffy coat obtained from blood was applied to Histopaque-1077 (Sigma Chemical Co., St. Louis, MO, USA) and centrifuged at 500 g for 30 min at room temperature (Chaturvedi *et al* 1979). The mononuclear cell fraction was collected, washed with Hank's balanced salt solution (HBSS), suspended in minimum essential medium (MEM)-HEPES containing 5% foetal calf serum (FCS) and antibiotics at a concentration of 5×10^6 cells/ml and layered in glass Petri dishes for 1.5–2 h at 37°C in humidified atmosphere of 5% CO₂. More than 90% of the adherent cells were macrophages as judged by morphology and latex particles phagocytosis. The bottom layer consisted of polymorphonuclear cells, which was collected separately. More than 95% of these cells were neutrophils on the morphology basis, which was studied in Leishmann's stained smears. The cells were washed and suspended in HBSS. Cell viability was checked by Trypan blue dye exclusion method.

2.3 Preparation of hMDF in vitro

The monocytes (5×10^6 viable cells/ml) in MEM-HEPES with 5% FCS were stimulated with 10^2 LD₅₀ of purified JEV infected adult mouse brain suspension and cultured for 2 h at 37°C in the presence of 5% CO₂. The adherent cells were washed thrice with phosphate buffered saline (PBS) and incubated with normal saline (NS) for 24 h at 37°C in the presence of CO₂. The supernatant was collected, centrifuged at 2000 g for 15 min and tested for neutrophil chemotactic activity. The 24 h culture supernatant of monocytes stimulated with normal mouse brain suspension (10% w/v) was simultaneously prepared to serve as control.

The crude supernatants from cultures of JEV-stimulated human peripheral monocytes were concentrated by freeze drying in Speed Vac (Savant Instruments Inc., New York).

The concentrated supernatant was purified on Superose-12 fast protein liquid chromatography (Pharmacia, Uppsala, Sweden). The fractions obtained were tested for neutrophil chemotactic activity. The protein content was estimated by the technique of Lowry *et al* (1951). The chemotactic fractions were concentrated before subjecting to SDS-PAGE for molecular weight determination, in which hMDF migrated as a single ~ 10 kDa band (Khanna *et al* 1991). It reacted specifically with anti-hMDF antibodies in Western blot and Dot-blot tests (Singh *et al*, unpublished results).

2.4 Assay of neutrophil chemotaxis

Neutrophil chemotaxis was assayed as described by Khanna *et al* (1991). Briefly, purified human peripheral neutrophils (1.5×10^6 /200 µl) were taken in upper chamber of multiwell chemotactic chamber (Millipore Inc., USA), separated from the lower compartment by a 5 µ pore size nitrocellulose filter. The lower compartment was filled with samples to be screened for chemotactic activity, which included macrophage culture supernatants stimulated with either JEV or normal mouse brain suspension, while HBSS served as negative control. N-formyl-methionyl-leucyl-phenylalanine (FMLP, 10^{-7} M) was used as a positive control. After incubation at 37°C in 5% CO₂ atmosphere, the filters were removed, fixed in 70% isopropyl alcohol and stained with haematoxylin. The number of neutrophils migrated into the filter was counted in 5–7 randomly selected high power fields ($\times 400$). The samples were tested in triplicate and mean \pm SD was calculated.

2.5 Preparation of anti-hMDF antiserum

The anti-hMDF antiserum was prepared in 4–6 weeks old inbred Swiss albino mice as described by Khanna *et al* (1997). hMDF protein (100 µg) emulsified in Freund's complete adjuvant (Sigma) in 1 : 1 dilution was injected intramuscularly (i.m.) on the inner side of the flanks and the dose was repeated after 3 weeks, emulsified in 1 : 1 dilution with Freund's incomplete adjuvant (Sigma) intramuscularly. Following this, 3 intradermal (i.d.) injections (60 µg hMDF protein/mouse) were given in the abdominal area at weekly intervals, without any adjuvant, at 4–5 places. This was followed by an intravenous (i.v.) injection (40 µg hMDF protein/mouse) 4 days before bleeding. The mice were then bled by cardiac puncture, serum was separated and inactivated at 56°C for 30 min. The optimal dilution of antibody, which abrogated the chemotactic activity of hMDF was determined and stored at –70°C.

2.6 Study group

Eighty-two patients admitted with acute encephalopathic illness (acute, non-transient alteration of consciousness with or without fever, or other neurological symptoms) in Gandhi Memorial and Associated Hospitals, Lucknow were

enrolled for the study. A proforma directed history was taken and examination performed on admission. A careful record of patient's progress in hospital was maintained. JEV infection was confirmed by either isolation and identification of virus from cerebrospinal fluid (CSF) or its detection in CSF cells using indirect immunofluorescence technique (Mathur *et al* 1990) or by measuring JEV specific IgM antibody in acute CSF or 4-fold or greater rise in JEV specific HAI antibody titre in the serum (Sharma *et al* 1991). Acute phase serum was collected from all the patients on admission to the hospital and convalescent phase sera after an interval of 8–10 days. One or more indicators of JE infection were present in 26 of the patients. Control group consisted of serum samples from 20 normal healthy individuals.

Out of 26 JE confirmed patients included in the study, six (23.08%) died within a week of admission, six (23%) patients showed prolonged illness, while 14 (53.8%) recovered completely.

2.7 ELISA

ELISA for hMDF was standardized by the modified method of Voller *et al* (1976) for detection of hMDF and hMDF-specific antibodies. The assay was carried out as follows:

Flat-bottom microtitre plates (Nunc-Immuno Plate, Denmark) were coated with different concentrations of hMDF, ranging from 30 µg down to 23 ng and incubated in humidified atmosphere in different conditions viz., at 4°C or 37°C for 1 h or overnight to optimize the conditions for coating. The plates were washed and blocked for 1 h. After washing, anti-hMDF antisera (hMDF-As) diluted 1/500 was added to each well for 1 h at 37°C. For control, serum from sham-immunized mice and PBS were used in place of hMDF-As. Protein A conjugated with horse raddish peroxidase (HRP), diluted 1/10,000 in PBST was used as conjugate and *O*-phenylene-diamine as substrate. Sulphuric acid was added to stop the reaction and absorbance was measured at 492 nm on a Titertek Multiscan Plus ELISA reader (Labsystems, Finland). Absorbance values were either used directly or the absorbance values of the standard sample were used to construct a standard curve of arbitrary units. It was observed that coating of plates at 37°C overnight resulted in maximum absorbance development when compared with those obtained at other temperatures.

An inhibition ELISA for detection of hMDF in test samples was also developed. The plate was coated overnight at 4°C with hMDF-As (diluted 1: 500 in PBS). After washing and blocking as described above, test samples were added and incubated at 37°C for 1 h. After washing, protein A conjugated with HRP was added and the color was developed as described above. Absorbance was measured at 492 nm. Inhibition was calculated as follows:

$$\text{Inhibition (\%)} = 100 - \frac{A - b^1}{B - b^2} \times 100.$$

Where A is the absorbance of test sample, B is the absorbance where only PBS is added in place of test sample, b^1 is the blank of the test sample and PBS where anti hMDF-antisera not coated on the solid phase, and b^2 is the blank using PBS in uncoated wells. All the serum samples tested for hMDF in ELISA were confirmed by Dot-blot and Western blot assay also (results not shown).

2.8 Statistical analysis

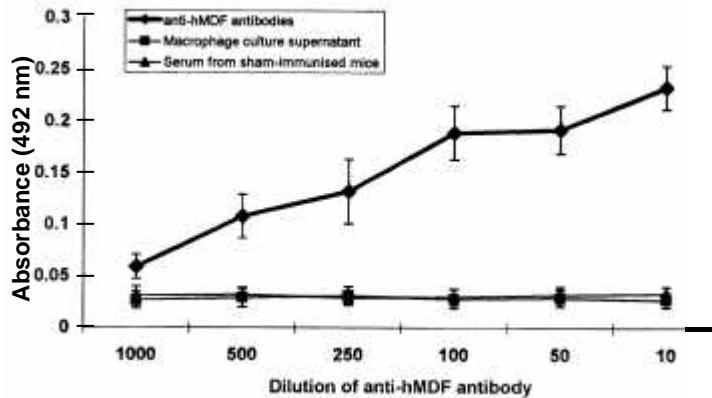
Every test was set up in triplicate and repeated 3 to 5 times. The mean value \pm SD from 9 to 15 values has been presented. The indices of sensitivity, specificity and accuracy of the established ELISA were calculated as follows:

Sensitivity = $\{(a/a + c) \times 100\}$; specificity = $\{(d/b + c) \times 100\}$ and accuracy = $\{(a + d)/(a + b + c + d)\} \times 100$ where a is the number of true positive samples, b is the number of false positive samples, c is the number of false negative samples and d is the number of true negative samples (Appassakij *et al* 1987). The samples positive in ELISA but negative in Dot-blot and Western blot were considered false positive; and samples negative in ELISA and positive in Dot-blot and Western blot were considered false negative.

3. Results

3.1 Evaluation of detection limit of hMDF-specific antibody

A checker board titration was done to find out the minimum amount of hMDF that reacted with minimum amount of anti-hMDF antibody by coating the plate with different concentrations of hMDF ranging from 30 µg down to 6 ng. The cut off value for optimum dilution of antibody was calculated by adding $2 \times$ SD with the mean absorbance obtained from the control. Macrophage culture supernatant



CONCENTRATION OF 37.5 µg/ml hMDF WAS SELECTED FOR FURTHER DEVELOPMENT OF THE hMDF-SPECIFIC ELISA. THE PLATES WERE COATED WITH hMDF SPECIFIC ANTIBODY WITH hMDF AT 37°C OVERNIGHT AND DEVELOPED AS DESCRIBED IN §2.7. MACROPHAGE CULTURE SUPERNATANT STIMULATED WITH NORMAL MOUSE BRAIN AND SERUM FROM

hMDF antibody. The absorbance values obtained were used directly to estimate the amount of hMDF-specific antibody in the test serum.

3.2 ELISA for detection of hMDF

An inhibition ELISA was developed for detection of hMDF in test samples by coating anti-hMDF antibodies on the solid phase as described in §2.7. Macrophage culture supernatant stimulated with normal mouse brain suspension and serum from sham-immunized mice were included in the assay as controls and per cent inhibition in absorbance values of the test samples and the controls was calculated. A standard curve presented in figure 2 was established by assaying purified hMDF preparation. It was observed that a concentration range of 3000 to 23 ng of hMDF could be detected by the developed ELISA. The curve was also used to detect hMDF quantitatively in test samples. The sensitivity and reproducibility of the established ELISA were estimated from this dose response curve.

3.3 Comparison of sensitivity of ELISA with chemotactic activity

A comparison of the sensitivity of ELISA test with neutrophil chemotaxis assay was made by titrating various amounts of purified hMDF. Findings summarized in figure 3 demonstrate that with the neutrophil chemotactic activity assay, the minimum amount of hMDF that could be detected was about 500 ng; while hMDF ELISA was significantly reactive up to a concentration of 23 ng (figure 2). Various types of preparations were assayed to test this system as follows.

3.4 Detection of hMDF in JEV-stimulated human peripheral blood mononuclear cell subpopulations

Cultures of normal human peripheral blood cells or its enriched subpopulations were stimulated with JEV *in vitro*. Control cultures were simultaneously prepared by stimulating the cells with normal mouse brain suspension. The culture supernatants were collected daily from 24 to 72 h and assayed for the presence of hMDF. An inhibition ELISA was done to detect the presence of hMDF in supernatants of JEV stimulated and control cultures. Figure 4 shows a peak inhibition of $26 \pm 4\%$ at 24 h in supernatants of total peripheral blood leucocyte cultures and of $31 \pm 5\%$ in macrophage enriched culture supernatants. The inhibition in supernatants from total cells as well as in macrophage cultures decreased gradually in 48 and 72 h in comparison to that of 24 h. T-cells and B-cells enriched culture supernatants had no significant activity of hMDF.

3.5 Detection of circulating hMDF in JEV-infected mice

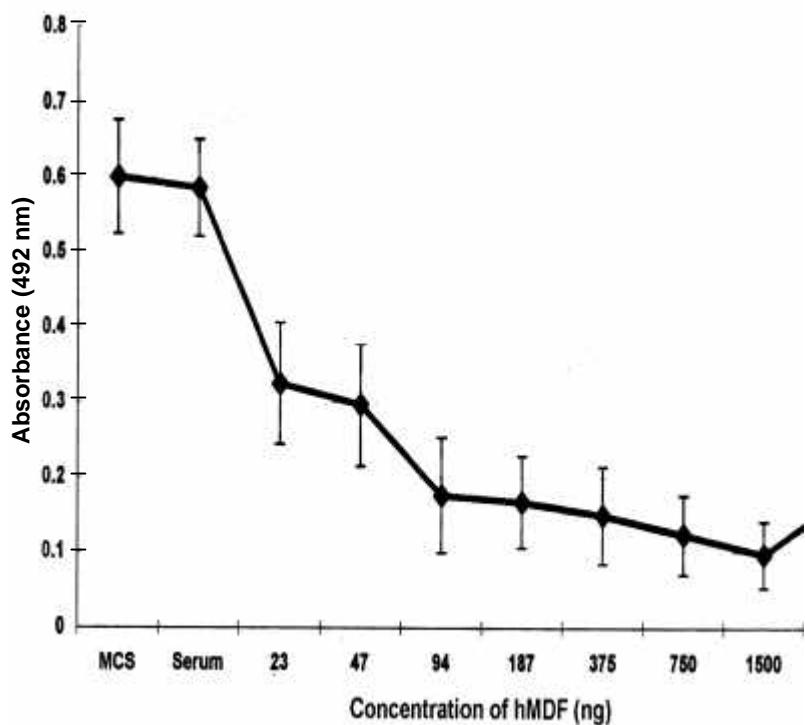


Figure 2. An inhibition ELISA for detection of chemotactic factor (hMDF). Plates were overnight at 4°C with anti-hMDF antibodies (1:500 in PBS). Diluted concentrations of hMDF were for 1 h at 37°C. Absorbance was measured at 492 nm and per cent inhibition calculated as in §2.7. Macrophage culture supernatant (MCS) stimulated with normal brain was included as hMDF

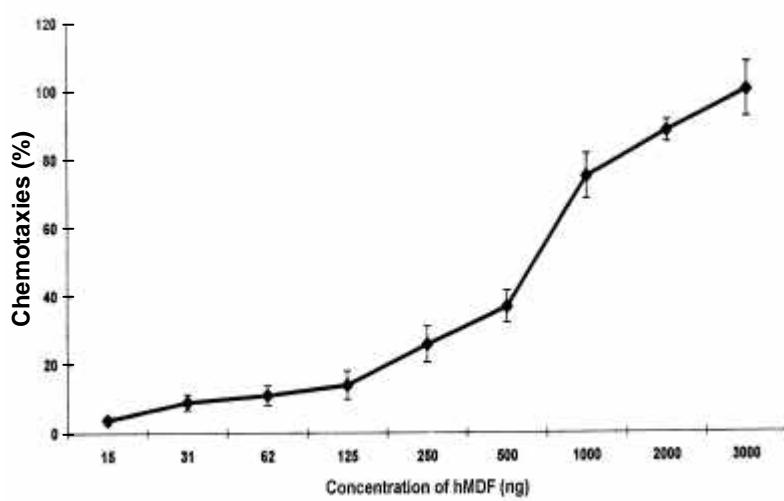


Figure 3. Titration of chemotactic factor (hMDF) by neutrophil chemotactic activity assay (◆) by modified Boyden chamber technique as described in §2.4, using different concentrations of hMDF. Each sample was tested in triplicate with neutrophil migration counted in 5-7

these mice till 100% mortality was observed. The serum was separated and screened for the presence of neutrophil chemotactic activity by inhibition ELISA. Serum from mice injected with normal mouse brain suspension was used as control. Results presented in figure 5 show that significant inhibition in hMDF-ELISA i.e., appearance of significant amount of hMDF commenced from day 4 and peaked by day 6 indicating maximum amount of circulating hMDF

when 100% mortality was observed.

3.6 Detection of hMDF in human sera

To detect the presence of hMDF in circulation, an inhibition ELISA was performed in sera of 26 JE confirmed patients. All 26 acute and 20 convalescent sera were screened for the presence of hMDF. Twenty normal human sera were used

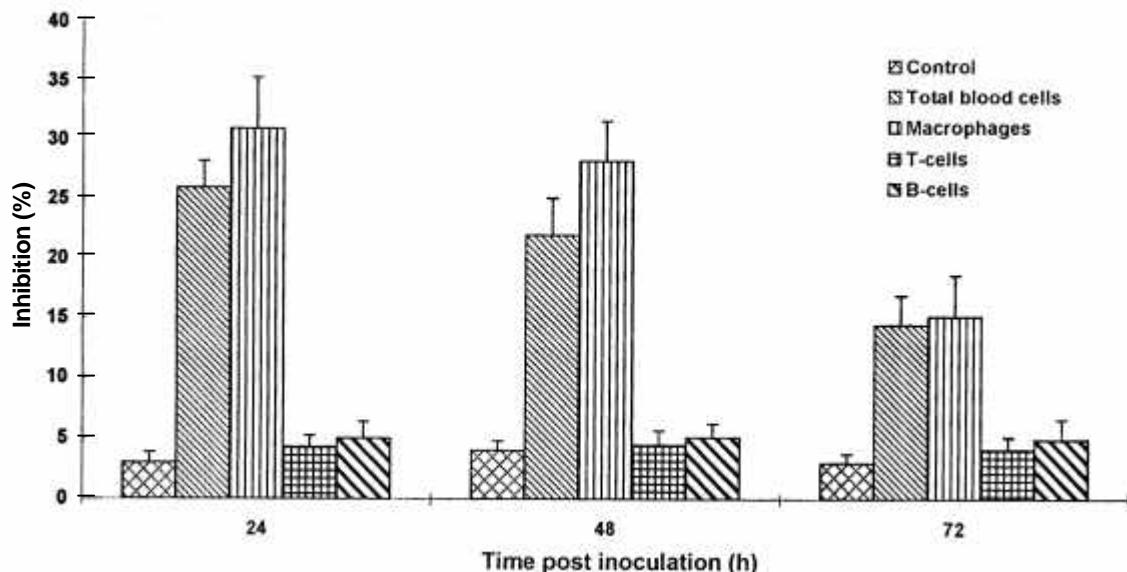


Figure 4. An inhibition ELISA to detect the presence of chemotactic factor (hMDF) in culture supernatants from *in vitro* JEV-stimulated human peripheral blood mononuclear cells (PBMC), or its enriched subpopulation of macrophages, T-cells or B-cells. Controls were inoculated with normal mouse brain (NMB) and uninoculated cultures were put up for blank. The test was repeated thrice and mean \pm SD

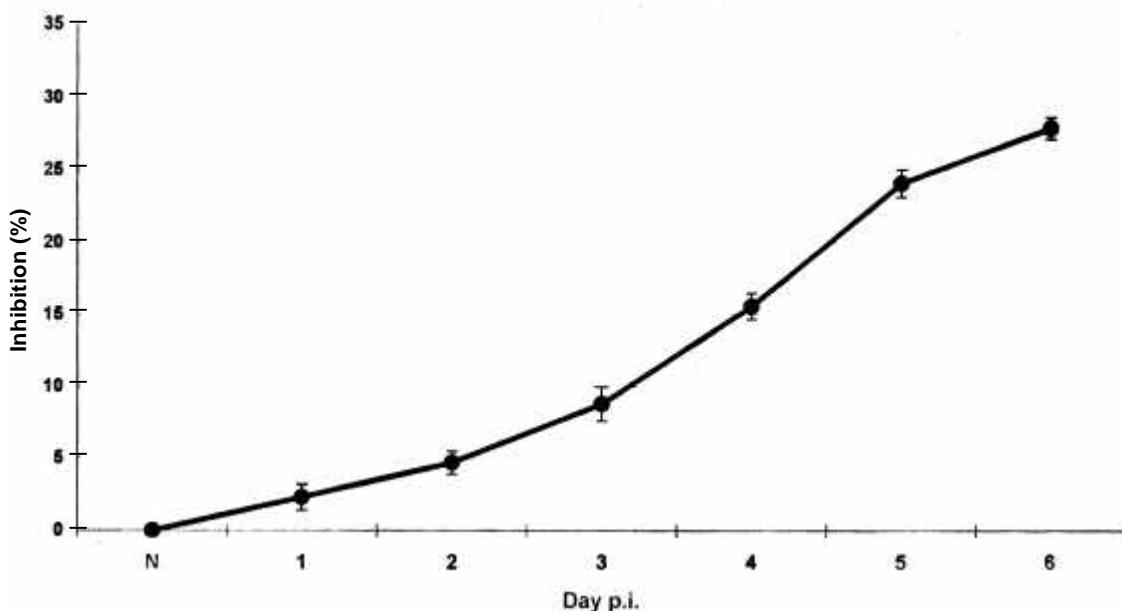


Figure 5. Inhibition ELISA for detection of chemotactic factor in sera of JEV-infected mice at different days post infection. Sera from mice infected with normal mouse brain suspension (N) were used as control. Blood, collected daily from both groups of mice was screened for the presence of chemotactic factor. Mean absorbance from triplicate tests was used to calculate the per cent inhibition.

as control. The cut off value for positivity of each test serum was calculated by adding $2 \times \text{SD}$ to the mean inhibition value of the control sera. The results presented in figure 6 show that 22 acute (84.6%) and 6 convalescent (30%) sera from cases of JE had significant inhibition value on hMDF-ELISA indicating the presence of hMDF like protein in circulation. All 6 patients, who showed high level of hMDF in second sample, were seriously ill at the time of sample collection.

The sensitivity, specificity and accuracy of the developed hMDF ELISA were also calculated. It was observed that the described ELISA is reproducible and the specificity, sensitivity and accuracy of the hMDF-inhibition ELISA were found to be 91%, 89% and 92.2% respectively.

4. Discussion

In this study, the development of a sensitive ELISA method for the determination of human monocyte-derived neutrophil chemotactic factor (hMDF) and its antibodies in the test samples and demonstration of hMDF in sera of JE confirmed patients is described. Cytokines play key roles in a number of host defense reactions (Baggiolini 1998). Among cells responding to chemokines, monocytes respond to the widest among of mediators (Mantovani 1999). Variations in the amount or quality of any chemokine or its receptor would have bearable consequences for basal trafficking of phagocytes. But now, they are also attracting much attention as pathogenic or marker substance in

various diseases (Ida *et al* 1992). Accurate quantification of such cytokines in body fluid samples is necessary for further investigation of their relationship to various diseases. This quantification is mostly performed by ELISA (Ledur *et al* 1995), which has been described extensively (Tsang and Weatherbee 1996; Beech *et al* 1997; Jung *et al* 1998). The only method for the detection of hMDF activity till now is the chemotactic assay using normal mouse peritoneal neutrophils, by which the level of hMDF cannot be quantified. Also, a minimum of about 500 ng/ml of hMDF is required to give a positive reaction, while it was observed in this study that with ELISA the minimum detection limit of hMDF is up to 23 ng/ml, which is much lower than the other known detection assay.

Many proinflammatory cytokines including IL-8 have been shown before neutrophil influx e.g., during bacterial meningitis (Lopez-Cortes *et al* 1995) and aseptic meningitis (Ishiguro *et al* 1997). The role of neutrophils in providing first line of defense against bacterial (Benveniste 1992) and viral (Srivastava *et al* 1999) infections has been demonstrated. We have shown the production of hMDF from human peripheral blood monocytes *in vitro* upon stimulation by JEV (Singh *et al*, unpublished results), causing neutrophil chemotaxis. hMDF is a low molecular weight (10 kDa), heat and pH resistant protein. In the present study, it was again confirmed by inhibition ELISA. The maximum activity of hMDF was observed at 24 h. JEV stimulated B or T-cell enriched subpopulations failed to produce any such chemotactic protein.

Serum samples from 26 JE confirmed patients were

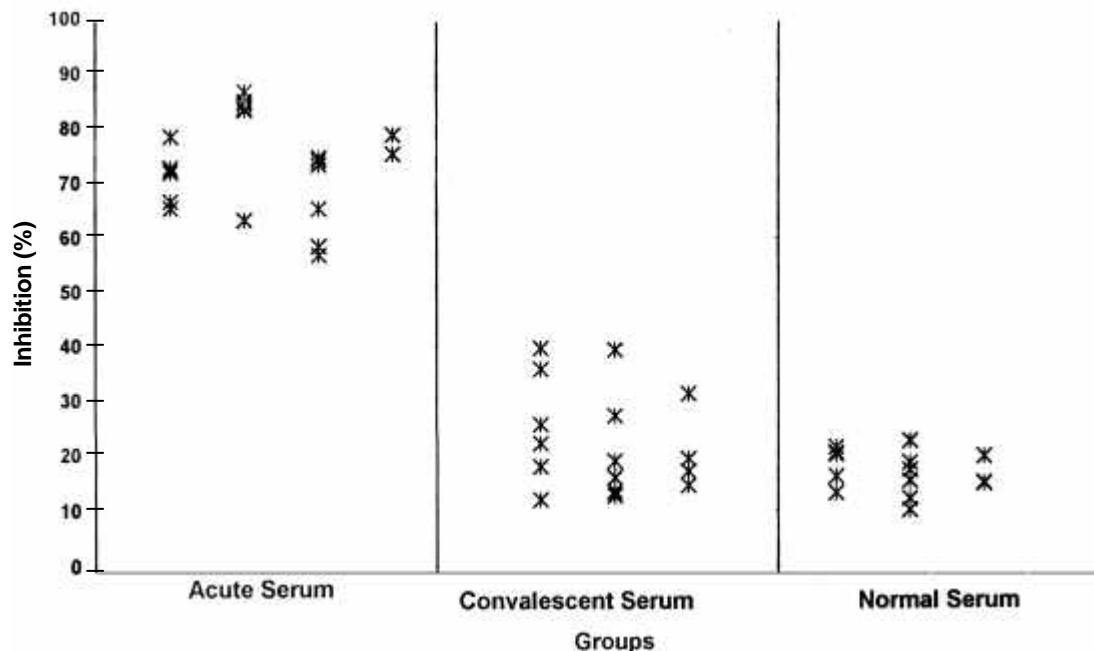


Figure 6. Detection of hMDF antigen in circulation of JE confirmed patients by inhibition ELISA. Twenty-six acute and twenty convalescent phase sera from JE confirmed patients were screened for neutrophil chemotactic factor. Mean $\pm 2 \text{ SD}$ from 20 normal human sera was taken as cut off value.

collected and assayed for hMDF by ELISA. The study revealed that hMDF and its antibodies could be easily quantified in human samples by the described ELISA. The reported ELISA is sensitive, simple and is directly applicable to clinical specimens as compared to the other known assay. MAC-ELISA positivity in the test group was found to be 77% (20/26 CSF showed the presence of IgM antibodies), while hMDF ELISA could detect anti-hMDF antibodies in 84.6% of the acute serum. The reported technique tells about the prognosis also and is preferred in those cases where CSF is not available. In a number of immune disorders (bacterial, fungal and viral infections) and for inflammatory diseases (arthritis, non-septic shock etc.), cytokine levels can be of great importance for diagnosis and therapeutic treatments (Ledur *et al* 1995). The role of chemokines in a wide range of inflammatory diseases and host immune response makes them useful targets for therapeutic intervention (Liles and Van Voorhis 1995; Gillis and Williams 1998). Observations of neutralizing antibodies in animal models of inflammation are promising (Adams and Lloyd 1997). Sekido *et al* (1993) have shown that anti-IL-8 antibody is highly effective in a rabbit model of ischaemia reperfusion injury in the lung.

hMDF is a unique protein produced during JEV infection only, therefore its use for rapid diagnosis is possible. Because of the high sensitivity, the ELISA is expected to be effectively used for further investigation on the involvement of hMDF in pathogenicity during JEV infection. Finally, complete information on the structure, pattern of expression and functional roles of hMDF will clarify the full potential of its use for therapeutic applications.

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