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Breakdown of blood–brain barrier by virus-induced cytokine during Japanese encephalitis virus infection

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Summary. In this study we have shown, for the first time, that Japanese encephalitis virus (JEV) and a low molecular weight (10 kDa) macrophage-derived neutrophil chemotactic factor (MDF) produced following JEV infection in mice could cause an alteration in the permeability of the blood–brain barrier resulting in leakage of plasma protein bound Evans blue dye and radiolabelled erythrocytes in brain. The maximum leakage occurred at day 6 after intracerebral (i.c.) JEV infection and was sensitive to anti-JEV antisera. Further, MDF caused peak leakage of dye and radiolabelled erythrocytes at 1 h post inoculation with a decline thereafter. Complete restoration of the integrity of the blood–brain barrier occurred by the 4th hour. The extent of leakage was dose dependent and showed a direct correlation between the level of MDF, clinical sickness and virus titre in brain. Anti-MDF antisera protected the mice against the effects of MDF. These findings show that JEV-induced cytokine, MDF, alters the integrity of the blood–brain barrier and thus controls the cellular and plasma leakage into the CNS.

Keywords: macrophage derived factor, JEV, chemotactic factor, breakdown of blood–brain barrier, neutrophilia

Japanese encephalitis (JE) is a serious central nervous system disease commonly resulting in high mortality in children. Congestion, oedema and haemorrhage are predominant findings in the brain during the acute stage of infection (Monath 1990; Hase *et al*, 1990). Histological examination of brains revealed neuronal degeneration, glial nodule formation and perivascular inflammation. The inflammatory cells predominantly consist of T lymphocytes, macrophages and neutrophils (Johnson *et al.* 1985). Clinical encephalitis develops in only 1 in 300 persons infected with Japanese encephalitis virus (Halstead 1981). Although the presence of JEV has been demonstrated in vascular endothelial cells, the mechanism of invasion of the CNS is not clear. It has been postulated that specifically sensitized T cells enter the brain and release certain lymphokines which then attract the macrophages (Johnson 1971). However, not much is known about the cytokine/chemical mediators which induce alteration of the blood–brain barrier during JEV infection.

Recently, we have observed production of

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low MW (10 kDa) neutrophil chemotactic peptide from the splenic macrophages of JEV-infected mice. It is resistant to heat and acid or alkaline pH. The macrophage derived factor (MDF) is sensitive to prolonged incubation with proteases (Khanna *et al.* 1991) having a variety of in-vivo biological effects (Bharadwaj *et al.* 1991). The present study was undertaken to evaluate the role of MDF in the pathogenesis of JE and to investigate its effect on the integrity of the blood–brain barrier. The findings show that MDF produced during JEV infection controls cellular and plasma leakage through blood vessels which results in inflammatory response, cerebral oedema and haemorrhage.

Materials and methods

Virus

JEV strain 78668A isolated from human brain in India was passed five times intracerebrally (i.c.) in suckling Swiss albino mice (Mathur *et al.* 1981) and was used as a 10% (w/v) infected mouse brain suspension. The infectivity titre of virus in suckling mice was 4.6 LD_{50}/0.025 ml. The 78668A strain has been sequenced by Chen *et al.* (1990).

Preparation of macrophage derived factor

The macrophage derived factor (MDF) was prepared from splenic macrophages of JEV-infected Swiss albino mice as described previously (Khanna *et al.* 1991). Briefly, the mice were primed with a single intraperitoneal injection of 0.3 ml of 100 LD_{50} of JEV. On day 7 post-infection the spleens were harvested and the glass-adherent cells were cultured in saline. The 24-h macrophage culture supernatant was collected, concentrated in a Speed Vac (Savant Instrument Inc., USA) and purified on a Sephacryl S-200 column (Pharmacia) and migrated as a single 10 kDa band on SDS–PAGE. The chemotactic activity was assayed using a modification of the Boyden technique described by Pohajdak *et al.* (1986) using 5-μm pore size nitrocellulose filter. 10^{-7} M *N*-formyl-methionyl-leucyl-phenylalanine (Sigma) was used as a positive control. The number of neutrophils which had migrated into the filter at the end of 1 h incubation was counted in five to seven randomly selected high power fields ($\times 400$).

Assay of permeability of the blood–brain barrier

The blood–brain barrier permeability was measured by two different methods. In the first the leakage of protein in brain was assayed by measuring the release of plasma protein bound Evans blue dye into brain substance. Mice were inoculated into the tail vein with 200 μl of a 2% (w/v) solution of Evans blue in normal saline and MDF was given 5 min later. The mice were anaesthetized one hour later with halothene and were perfused with 10 ml saline through the left cardiac ventricle (Thumwood *et al.* 1988). A 10% homogenate of the brain was prepared in normal saline and centrifuged at 6000 r.p.m. for 15 min. The OD of the clear supernatant was determined at 590 nm in a spectrophotometer. A standard curve was drawn with various concentrations of bovine serum albumin (Lowry *et al.* 1951) and the protein content of each brain extract was determined by comparing the OD units with it. The results were calculated as μg protein/brain and expressed as mean $\pm$ s.e. of 10–12 mice. In the second method, the movement of radiolabelled erythrocytes was assessed. Fresh mouse erythrocytes were washed with Hanks balanced salt solution (HBSS). 3×10^9 r.b.c./ml were incubated with chromium-51 (1 mCi/ml as sodium chromate, Bhabha Atomic Research Centre, India) for 30 min at 37°C and then washed. 100 μl of cell suspension (3×10^9 cells/ml) was injected i.v. into mice followed by MDF i.v. 5 min later. Mice were anaesthetized after 1 h and brains were removed after perfusion with 10 ml of saline as described above. Whole brains were scanned for ^{51}Cr contents using an LKB Mini Gamma counter. Mice injected with

normal mouse brain suspension or normal macrophage culture supernatant were used as controls. The values are expressed as c.p.m./brain as compared to that in control mice. Data obtained from 10 to 12 mice are expressed as mean values ± s.e.

Measurement of oedema

The oedematous swelling in brain during JEV infection was measured by the method described by Mohanty *et al.* (1980). The brains from age and sex-matched JEV-infected and control groups of mice were weighed. For the measurement of dry weight, the brains were dried in an oven at 50°C until the weight became constant. The percentage oedema was calculated as described by Rapopart (1976).

Indirect immunofluorescence

Serial transverse imprint smears of brain were prepared and screened for JEV specific antigen by indirect immunofluorescence as described previously (Mathur *et al.* 1989). The anti-JEV monoclonal antibody designated 98.9.5i was kindly provided by Dr E.A. Gould, Oxford, UK.

Preparation of MDF-specific antiserum

The anti-MDF antiserum was prepared in rabbits. 100 μg of MDF emulsified in

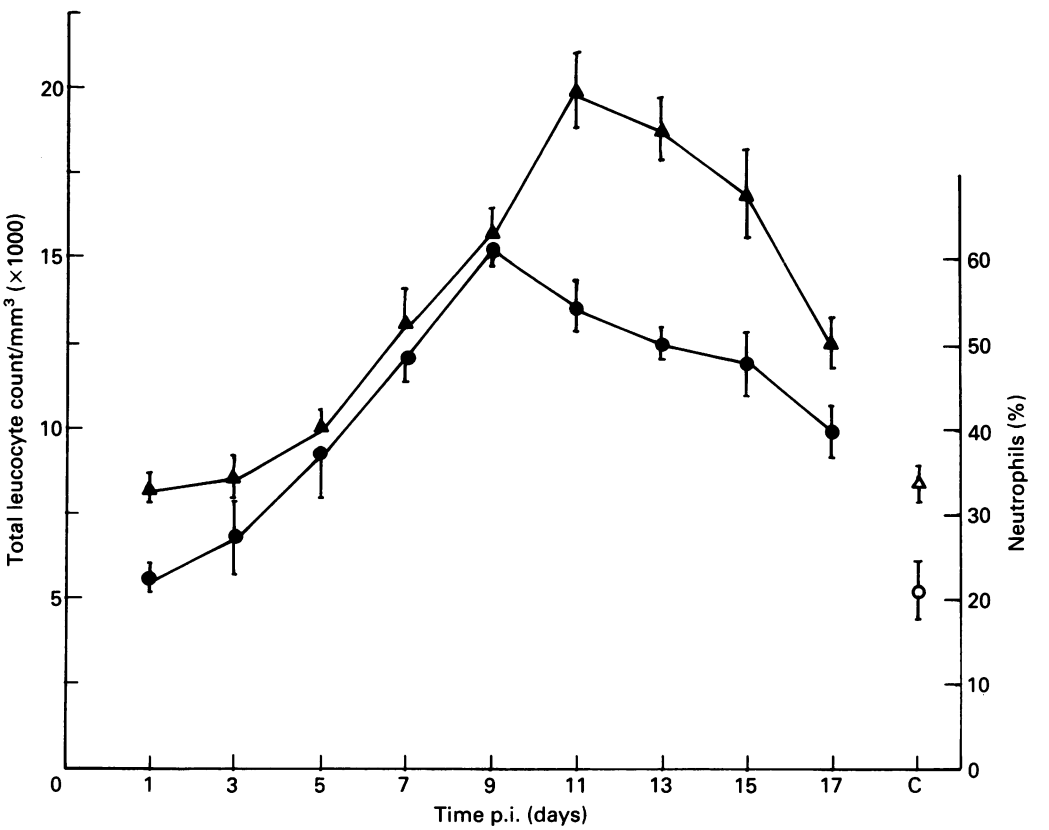

Fig. 1. ▲, Total leucocyte counts and ●, percentage neutrophils at different periods in the peripheral blood of JEV i.p. inoculated mice. Each value represents mean ± s.e. from five mice.

Freund's complete adjuvant was injected subcutaneously at four different sites in rabbit and this dose was repeated at weekly intervals for 6 months. The rabbits were then bled from the ear vein and sera were collected and inactivated at 56°C for 30 min. The optimal dilution of the antibody which abrogated the chemotactic activity was determined and stored at −70°C until used. Anti-JEV antiserum was provided by the National Institute of Virology, Pune, India.

Results

JEV infection in mice

Intracerebral inoculation of JEV in inbred Swiss albino mice produced clinical illness such as arching of the back, convulsion or paralysis from day 4; all the mice died by day 6. Imprint smears of brain following JEV infection of the mice showed large numbers of immunofluorescence positive cells in thalamus and brain-stem. The infectivity titres of JEV in brain from these mice were assayed daily. There was a gradual rise in infectivity titre in brain which was $10^{4.5}$ LD_{50}/0.025 ml by day 6 p.i. JEV given intraperitoneally (i.p.) produced no clinical illness or virus isolation from brain. The maximum virus titre in spleen after i.p. inoculation of JEV was $10^{2.1}$ LD_{50}/0.025 ml on day 7.

Effect of JEV on leucocyte counts

Figure 1 shows the total leucocyte counts at different periods following intraperitoneal inoculation of JEV. JEV infection induced

Table 1. Cerebral oedema in mice after JEV inoculation

Groups of mice	Day p.i.	Weight of brain (mg) wet	Weight of brain (mg) dry	Water content (%)	Swelling (%)
After i.c. inoculation					
JEV infected	2	304±13	72±1	76	—
Control		300±16	71±2	76	
JEV infected	4	330±18	75±0.7	79	9.5
Control		320±15	73±4	77	
JEV infected	5	367±17	73±3	80	15
Control		325±9	72±1.3	77	
After i.p. inoculation					
JEV infected	5	331±10	83±3	77	4.34
Control		328±8	78±2.1	76	
JEV infected	7	350±14	71±4	79	4.76
Control		338±13	74±1	78	
JEV infected	9	341±11	69±4	80	5
Control		335±9.8	68±5	79	

Mice were inoculated with 0.025 ml of 100 LD_{50} of JEV i.c. or with 0.3 ml of JEV i.p. Brains were collected at different days. Control mice received normal mouse brain suspension intracerebrally (i.c.) or intraperitoneally (i.p.). Data are presented as mean of 10 to 12 mice ± s.e.

leucocytosis with neutrophilia. The mean count in JEV-infected mice was 20000± 580/mm^3 on day 11 p.i. while in normal controls it was 8400±212/mm^3. There was a significant rise ($P<0.001$) in percentage neutrophils in JEV-infected mice (60±1%) compared with controls (21±3%).

Effect of JEV on cerebral oedema

Intracerebral injection of JEV in mice showed a gradual increase in weight of brain which remained significantly higher than that of control mice receiving normal mouse brain suspension. The mean value for brain weight in JEV-infected mice was 367±17 mg while in controls it was 325±9 mg ($P<0.001$) on day 5 p.i. The dry weights of brains remained almost the same in infected and control mice (Table 1). Thus, the rise in brain weight of JEV-infected mice can be attributed to oedematous swelling. The mice injected with 0.3 ml of 100 LD_{50} of JEV intraperitoneally showed slight increase in their brain weight as compared to controls (Table 1) thus accounting for mild pathological changes occurring in the brains of mice after i.p. challenge with JEV.

Effect of JEV on the blood–brain barrier

Groups of control and JEV-infected mice were inoculated with Evans blue daily and their brains were examined after perfusion with PBS. A deep blue colouration of the whole brain was observed on day 6 p.i. in JEV-infected mice. The protein bound dye leakage from capillaries to extra-vascular spaces in brain coincides with the cerebral symptoms. The control mice, which were given 0.025 ml of normal mouse brain suspension, did not show any dye leakage in brains (dye protein content 90±12 μg/mouse). The dye protein in brain homogenates of infected mice was 118±6 μg/brain up to day 3 p.i. followed by a sharp rise from day 4. On day 6 p.i. it was 235±20 μg/brain (Fig. 2b), thus giving varying degrees of colouration on different days. In contrast, the brains from intraperitoneally inoculated mice showed

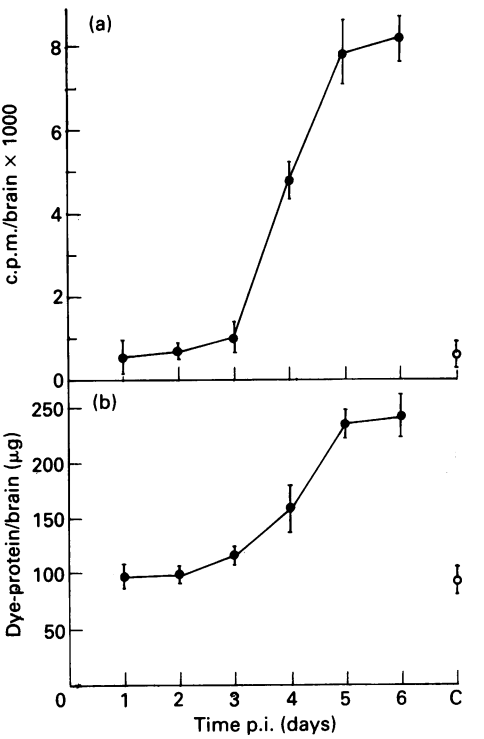

Fig. 2. a, ^{51}Cr labelled erythrocytes and b, leakage of protein bound Evans blue in the brain of mice on different days after i.c. JEV infection. Protein bound Evans blue leakage was determined spectrophotometrically. Whole brain was scanned in a gamma counter to determine the leakage of ^{51}Cr labelled erythrocytes, and c.p.m./brain is presented. Each value represents mean±s.e. from 12 mice.

very little blue colouration and dye leakage (150±10 μg/brain) on day 10 p.i.

The leakage of ^{51}Cr labelled erythrocytes from capillaries into brain tissue was assayed. The findings summarized in Fig. 2a show a gradual increase in the leakage of radiolabelled erythrocytes in the brain tissue from day 3 for JEV-infected mice while for control mice it was negligible.

Effect of anti-JEV antisera treatment on alteration in the integrity of the blood–brain barrier induced by JEV

Groups of mice were injected i.v. with 200 μl

of anti-JEV antisera, followed by i.c. challenge with 0.025 ml of 100 LD_{50} of JEV 24 h later. The doses of antisera were repeated on days 3 and 5 p.i. The effect of antisera treatment on the integrity of the blood–brain barrier was examined on day 6 p.i. by measuring the leakage of dye bound protein in brain. The findings showed that plasma leakage into brain was partially abrogated by pretreatment of mice with anti-JEV antisera (dye protein content 139 ± 18 μg/brain). Dye protein content from JEV-infected mice was 237 ± 20 μg/brain.

Macrophage-derived factor

We have identified a low MW (10 kDa) neutrophil chemotactic peptide secreted by splenic macrophages with peak activity on day 7 after intraperitoneal inoculation of JEV (Khanna *et al.* 1991). Therefore, the neutrophil chemotactic activity of MDF was screened on different days after i.c. or i.p. inoculation of JEV into mice. The findings showed that peak chemotactic activity of MDF appeared on day 5 following i.c. (mean value 32 ± 3.2 neutrophils/h.p.f.) and on day 7 following i.p. (value 34 ± 5.2 neutrophils/h.p.f.) routes of JE virus inoculation.

Effect of MDF on leucocyte count

The total and differential leucocyte counts in peripheral blood of mice inoculated with 2 or 5 μg of purified MDF (i.v.) were studied at different intervals. Distinct leucocytosis with neutrophilia (maximum $63 \pm 5\%$) was observed at 1 h after 5 μg of purified MDF inoculation (Table 2).

Table 2. Peripheral blood leucocyte counts in MDF inoculated mice

Time post inoculation (h)	Total leucocyte counts (per mm^3)	Neutrophil counts (%)
1	14980 ± 419	63 ± 5
2	13100 ± 575	59 ± 2
3	10228 ± 256	42 ± 1
Control	8600 ± 116	28 ± 2

The mice were injected with 5 μg of purified MDF i.v. Total leucocyte counts were done and smears were prepared from each mouse from tail vein at different intervals. The values are expressed as mean of 7–9 mice $\pm$ s.e.

Effect of MDF on the blood–brain barrier

Groups of mice were inoculated with 200 μl of Evans blue dye i.v. followed by 200 μg of purified MDF i.v. 5 min later and alteration in the integrity of the blood–brain barrier was assayed at different periods. Control mice were given normal mouse splenic macrophage supernatant in place of MDF. The findings show that a peak increase in leakage of dye in brain occurred at 1 h with deep blue colouration of brain. The protein leakage declined gradually and the integrity of the blood–brain barrier was restored by 4 h (Fig. 3b). Therefore, in further experiments observations were recorded at 1 h after inoculation of MDF. To investigate the leakage of erythrocytes from the capillaries, ^{51}Cr labelled erythrocytes were injected i.v. in groups of mice followed by injection of 200 μg of MDF i.v. The ^{51}Cr-erythrocyte counts in the brains from MDF injected mice were higher than that for control mouse brains (Fig. 3a).

The relationship between the concentration of MDF and dye leakage in the brain at 1 h post inoculation was screened. The findings presented in Fig. 4 show that with increasing dose of MDF a greater degree of dye leakage in brain was observed.

Effect of anti-MDF antisera treatment on protein leakage

Purified MDF (0.2 mg) was inoculated i.v. in groups of mice preinoculated with 0.2 ml of anti-MDF antisera (1:10 diluted) 24 h before. The leakage of protein bound dye in brain after anti-MDF antisera treatment was assayed and compared with control. A reduction in leakage of protein was observed

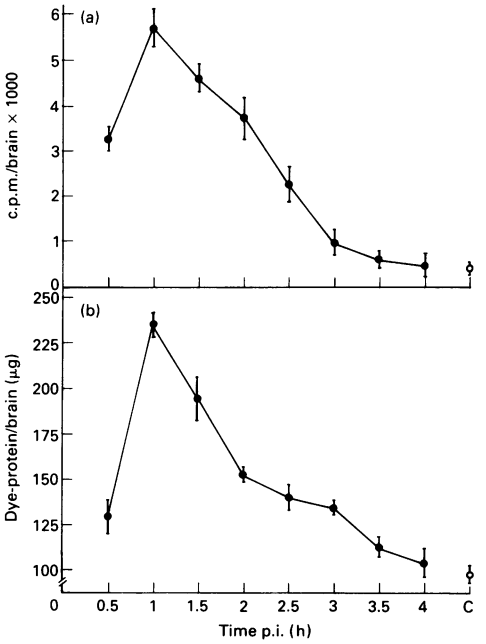

Fig. 3. a, Radiolabelled erythrocytes and b, leakage of dye bound protein in the brain of mice at different periods after MDF inoculation. Dye-protein leakage was determined spectrophotometrically. Whole brain was scanned in a gamma counter to determine the leakage of radiolabelled erythrocytes, and c.p.m./brain is presented. Each point represents mean ± s.e. from 12 mice.

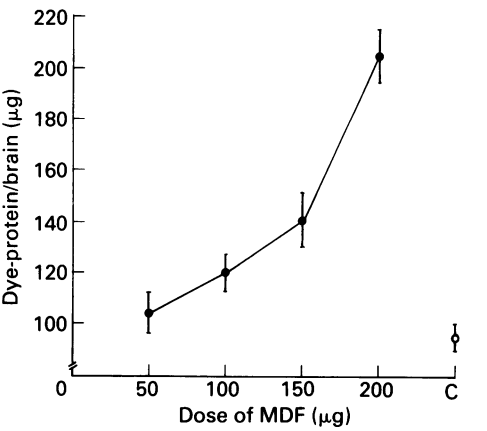

Fig. 4. Effect of different doses of intravenously inoculated MDF on plasma–protein leakage in brain tissue of mice. Values are presented as mean ± s.e. from 10 mice.

in anti-MDF antisera treated mice (116 ± 19 μg/brain) as compared to untreated mice (216 ± 13 μg/brain). The protein content of normal mouse brain was 99 ± 8 μg/brain.

Discussion

Japanese encephalitis virus, a mosquito borne flavivirus (Westaway *et al.* 1985), is one of the major causes of acute encephalitis, particularly in Asia (Umenai *et al.* 1985; Kumar *et al.* 1990). The mechanism by which the virus invades the CNS remains controversial, although both neural (Monath *et al.* 1983) and haematogenous (Johnson *et al.* 1985) routes have been suggested. In the present study we have attempted to characterize some of the effects of JEV infection in breaching the blood–brain barrier in mice and compare it *in vivo* with purified MDF.

The MDF acts as a chemoattractant and induces neutrophil leucocytosis in mice. We have previously reported marked neutrophil leucocytosis in the early phase of infection in patients of Japanese encephalitis (Chaturvedi *et al.* 1979). Following intraperitoneal inoculation of JEV, infiltration of macrophages along with polymorphonuclear cells has been observed in spleen of mice (Mathur *et al.* 1988) and coincides with MDF production. Chemotactic substances, such as leukotriene B_4 and C5a have been shown to induce neutrophil dependent plasma leakage in hamsters (Bjork *et al.* 1985) or rabbits (Wedmore & Williams 1981). IL-8, a low molecular mass cytokine (8–10 kDa) has been identified as mediator of neutrophil chemotaxis (Van Damme *et al.* 1989). It is produced by a variety of cells such as monocytes (Yoshimura *et al.* 1987), fibroblasts (Van Damme *et al.* 1989) and endothelial cells (Strieter *et al.* 1988). IL-8 activates neutrophils (Peveri *et al.* 1988) and can produce oedema (Colditz *et al.* 1989). Our earlier findings (Khanna *et al.* 1991) suggested that MDF produced during JEV infection in mice is a low MW (10 kDa) neutrophil

chemotactic factor. Peak activity of MDF appears at day 7 post JEV infection coinciding with the influx of neutrophils into the affected tissue (Mathur *et al.* 1988). N-terminal-amino-acid sequence analysis of MDF is in progress and further homology with related inflammatory proteins could be investigated.

Normally the blood–brain barrier prevents invasion of CNS by micro-organisms because of continuous vascular endothelium lining. The breach could occur because of damage of this endothelial lining by trauma, allergic or inflammatory lesions, or because of the release of vasoactive mediators. Our results demonstrate that during JEV infection or after MDF inoculation in mice, breakdown of the blood–brain barrier occurs resulting in leakage of dye bound protein and radiolabelled erythrocytes into the brain substance causing cerebral oedema and microhaemorrhages. The localization of JEV antigen in brain capillary endothelial cells has been observed (Johnson *et al.* 1985). It is likely that during this period breakdown of the blood–brain barrier helps in the entry of virus into the brain with consequent development of encephalitis. It is known that only one in 200 or 300 JEV-infected individuals develops encephalitis (Halstead 1981). A high incidence of JEV encephalitis has been seen in children (Mathur *et al.* 1982) in whom it may be easier for virus to traverse the blood–brain junction because they have a thinner basement membrane than do adults.

In the present study the leakage of proteins is not due to morphological damage as vascular integrity was restored by 4 h after MDF injection and administration of anti-MDF antisera protects it. Also, that electron microscopic examination of JEV-infected brain showed no evidence of damage to the vasculature (Hase *et al.* 1990) supports our findings. Cerebral oedema is a constant feature in JE (Monath 1990). It occurs due to the increased hydrostatic pressure in the capillary bed owing to the movement of fluid from capillaries into the brain substance. The present findings show an increase in fluid content of brain during JEV infection and after MDF administration in mice. The MDF produced during JEV infection acts on blood capillaries through the release of histamine (Khanna *et al.* communicated).

We have recently demonstrated that JEV-stimulated human macrophages secrete a chemotactic peptide similar to mouse MDF (unpublished data). It is, therefore, likely that such a factor is responsible for cerebral inflammatory response and oedema following JEV infection.

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