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Regulation of vascular permeability by macrophage-derived chemotactic factor produced in Japanese encephalitis

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Summary The vascular effects of Japanese encephalitis virus (JEV)-stimulated splenic macrophage-derived neutrophil chemotactic factor (MDF) were evaluated in mice. Intraperitoneal injection of MDF in mice resulted in a rapid increase in capillary permeability in a dose-dependent manner as assessed by leakage of intravenously injected radiolabelled albumin ([^{125}I]-albumin) or Evans blue dye. Intradermal inoculation of MDF in rabbits caused [^{51}Cr]-labelled neutrophil emigration and accumulation into injected sites. Peak plasma leakage and neutrophil infiltration were observed at 1 h following MDF inoculation, and plasma leakage was restored by 2.5 h. The increase in capillary permeability was sensitive to pretreatment of mice with avil and ranitidine (H1 and H2 histamine receptor blockers, respectively), resulting in abrogation of the response; indomethacin, a prostaglandin synthetase inhibitor, did not have any effect.

Key words: capillary permeability, histamine, Japanese encephalitis virus, macrophage-derived factor.

Introduction

Japanese encephalitis virus (JEV), a mosquito-borne flavivirus, is a major cause of acute encephalitis in Asia. During the acute stage of infection, brain congestion, oedema, haemorrhage, inflammatory infiltration and neuronal degeneration are characteristic histological features. Among the predominant inflammatory cells recruited into perivascular areas in the brain are T lymphocytes with few mononuclear and polymorphonuclear cells.[1] Peripheral neutrophil leucocytosis or infiltration of neutrophils in extraneural tissues and in cerebrospinal fluid following JEV infection has been reported in humans or in experimental animals.[2–4] The accumulation of fluid and the migration of neutrophils at the site of injury are attributed to the action of soluble chemical mediators, which probably act by altering vascular permeability. The increased capillary permeability in response to antigen stimulation could be due to the release of vasoactive mediators such as complement components, kinins, histamine, serotonin, LTB_4, prostaglandin or cytokines.[5,6] The precise mechanism of recruitment of inflammatory cells and oedema formation during JEV infection is not known.

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Previous observations have revealed the production of a low MW (10 kDa) macrophage-derived neutrophil chemotactic factor (MDF) following JEV infection in mice.[7] The *in vivo* studies suggest that MDF modulates the activation of neutrophils,[8] regulates granulocytosis, breaches the blood–brain barrier[3] and controls iron metabolism.[9] The present study investigated the effects of MDF on the microvasculature.

Materials and methods

Virus

JEV (strain 78668A) isolated from the brain of a patient with Japanese encephalitis[10] was used in the study. The infectivity titre of virus in brain pools of suckling mice was 4.5 LD_{50}/25 μL. It produced 100% mortality by day 6 following intracerebral (i.c.) inoculation while i.p. inoculation produced no clinically evident disease.

Preparation and purification of MDF

MDF has been shown to be produced by splenic macrophages of JEV-primed inbred Swiss albino mice.[7] Briefly, the mice were inoculated i.p. with 0.3 mL of 10 LD_{50} JEV-infected mouse brain suspension. Spleens were collected aseptically

on day 7 postinfection (p.i.). Splenic single cell suspension (1×10^7 cells/mL) in MEM-HEPES was seeded in glass Petri dishes for 2 h at 37°C in a 5% CO_2 atmosphere. The glass-adherent cells were washed with PBS (pH 7.4) and were further cultured in saline in glass Petri dishes for 24 h. The supernatant was collected, centrifuged and assayed for neutrophil chemotactic activity by a modified Boyden chamber technique[7] using 5 μm pore size nitrocellulose filter. *N*-formyl-methionyl-leucyl-phenylalanine (10^{-7} mol/L Sigma, MO, USA) was used as a positive control. MDF was concentrated by freeze-drying in a Speed Vac (Savant Instruments Inc., New York, USA) purified on Sephacryl S-200 column (Pharmacia, Uppsala, Sweden) using 0.1 mol/L PBS (pH 7.4). The chemotactic fractions were concentrated and protein content determined. Molecular weight and purity of these fractions was tested by sodium dodecyl sulfate-PAGE in linear gradient (10–18%) polyacrylamide gels. Control mice were injected with 0.3 mL of 10% normal mouse brain suspension. Mouse splenic macrophage supernatant was prepared on day 7 p.i. as described above.

Assay of capillary permeability

Alterations in vascular permeability by MDF were studied by measuring the leakage of plasma protein-bound Evans blue dye[11] or [^{125}I]-labelled BSA into the peritoneal cavity of mice. Radio-iodination of BSA was carried out by lactoperoxidase-catalysed reactions.[12] One hundred microlitres of a 2% (w/v) solution of Evans blue (E. Merck, Darmstadt, Germany) or [^{125}I]-preparation (1 μCi; Bhabha Atomic Research Centre, Bombay, India) were injected i.v. into the tail veins of mice followed by a 5 μg of MDF (in 100 μL of PBS) injection i.p. Mice were sacrificed at different periods and their peritoneal cavity was lavaged with 4 mL of saline. The peritoneal washings were made up to 10 mL and filtered through glass wool; they were screened for [^{125}I]-labelled albumin content on an LKB Gamma counter and expressed as ct/min per mL. Absorbance was recorded at 590 nm.[6] The dye-bound protein leakage was measured by matching the absorbance of peritoneal washings with the standard curve drawn with various concentrations (10–200 μg) of bovine serum albumin[13] and expressed as μg/mouse. Control mice received normal mouse splenic macrophage supernatant. The data have been subjected to Student's *t*-test for statistical evaluation.

Assay of neutrophil emigration

The neutrophil accumulation at the site of intradermal (i.d.) MDF injection was studied using [^{51}Cr]-labelled neutrophils.[14] More than 95% purified[15] allogenic rabbit neutrophils (5×10^7 cells/mL) were [^{51}Cr]-labelled (100 μCi/mL as sodium chromate; Bhabha Atomic Research Centre, by incubation at 37°C for 30 min, washed three times with Ca^{2+}- and Mg^{2+}-free Tyrodes' solution and resuspended in 2 mL of the same solution containing 10% autologous plasma and injected into the marginal ear vein of the donor rabbit. Eight groups of rabbits (n = 3–5 in each group) were injected with MDF and control normal mouse splenic macrophage supernatant (10 μg in 100 μL of saline) at different sites. Midway through each time period a blood sample was collected and the *in vivo* specific activity of neutrophils was calculated as follows:

$$\text{Blood neutrophil specific activity} = \frac{[^{51}\text{Cr}]\ \text{ct/min per mL blood}}{\text{No. neutrophils/mL blood}}$$

Groups of rabbits were sacrificed at 30 min, another at 60 min and so on. Their blood was collected. The test and control sites were punched and the radioactivity in the tissues was determined in an LKB-gamma counter. The total number of neutrophils accumulated in MDF-injected or control sites was calculated as follows:

$$\text{Neutrophils/site} = \frac{[^{51}\text{Cr}]\ \text{ct/min per site}}{\text{Blood neutrophil specific activity at midpoint of experiment}}$$

The absolute number of neutrophils per site is expressed as per million [^{51}Cr]-labelled neutrophils per mL of blood ± s.e.

Anti-MDF antisera studies

The anti-MDF antisera was prepared as described elsewhere.[3] Polyclonal anti-MDF antisera (1000 μg/200 μL) was injected intravenously 24 h before the injection of MDF and Evans blue dye. The peritoneal washings collected 1 h later were screened for dye-bound protein spectrophotometrically. Control mice received normal rabbit serum in place of anti-MDF antisera.

Results

Assay of capillary permeability after MDF inoculation

Groups of mice were inoculated with Evans blue dye or [^{125}I]-labelled BSA followed by i.p. inoculation of MDF. The mice were sacrificed at different times. The findings summarized in Table 1 show that MDF induced an increase in leakage of protein-bound dye and [^{125}I]-labelled BSA. Significant plasma leakage ($P < 0.001$) occurred 1 h after MDF injection; vascular integrity was restored by 2.5 h. No leakage of plasma proteins was detected in control mice. Therefore, in further experiments observations were recorded at 1 h after inoculation.

Table 1. Effect on capillary permeability at different periods of exposure to MDF.

Time post-inoculation (h)	[^{125}I]-content of peritoneal washings (ct/min per mL)	Dye-bound protein (μg/mouse)
0.5	3091 ± 198	42 ± 6
1	5128 ± 487*	87 ± 15*
1.5	4375 ± 635	59 ± 7
2	2276 ± 331	48 ± 9
2.5	956 ± 412†	22 ± 6†
Control	600 ± 150	14 ± 1

Groups of mice were injected i.v. with 100 μL of either [^{125}I]-labelled BSA (1 μCi) or 2% Evans blue solution followed by 5 μg of MDF i.p. or normal mouse macrophage supernatant. Peritoneal washings from both groups were tested for ^{125}I or dye–protein content at different periods. Data are presented as the mean of 9–12 mice ± s.d. The control value represents the mean ± s.d. of washings taken from 2–3 control animals at each of the time periods studied. *$P < 0.001$; †$P > 0.05$.

Effect of MDF injection on neutrophil emigration

In studying the MDF-induced skin inflammatory response, groups of rabbits were injected with 5×10^7 [^{51}Cr]-labelled neutrophils into the marginal ear vein followed by i.d. injection of 10 μg of purified MDF or normal mouse macrophage supernatant as control. Blood samples were collected at the mid-point and at the end of the experiment. Rabbits were sacrificed at 30 min intervals. Radioactivity of punched out skin sites was determined as ct/min/site. The data presented in Fig. 1 show the peak accumulation of neutrophils ($28.96 \pm 2.66 \times 10^{-5}$) 1 h after MDF injection.

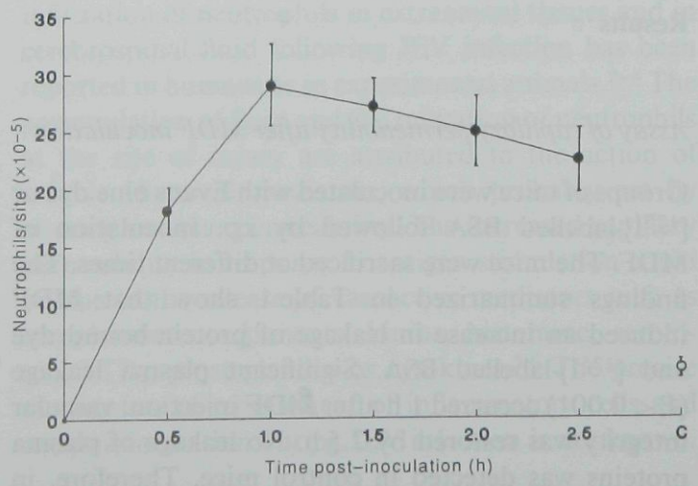

Fig. 1. [^{51}Cr]-labelled neutrophil accumulation at different periods after intradermal injection of MDF in rabbit.

Dose response

A linear relationship was observed between increasing dose of MDF and accumulation of [^{51}Cr]-labelled neutrophils or leakage of dye-bound protein (Fig. 2).

Effect of indomethacin treatment on MDF-induced alteration in capillary permeability

The present experiment was carried out to ascertain the role of prostaglandins in MDF-induced alterations in capillary permeability. Indomethacin, a prostaglandin synthetase inhibitor, was administered i.p. in mice

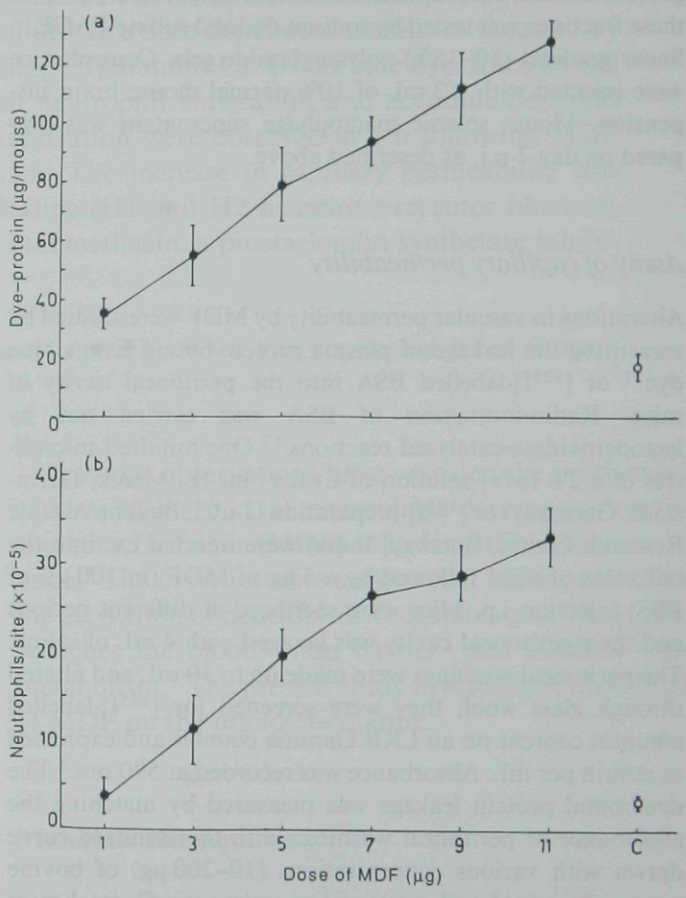

Fig. 2. (a) Effect of different doses of MDF on plasma protein leakage into the peritoneal cavity of mice. Groups of mice were injected with 100 μl of Evans blue dye followed 5 min later with different doses of MDF. Normal mouse splenic macrophage supernatant was used as control. Dye protein leakage was assayed 1 h later spectrophotometrically. Each point represents the mean ± s.e. from 10–12 mice. (b) Neutrophil accumulation after i.v. injection of 5×10^7 [^{51}Cr]-labelled neutrophils into rabbit followed by i.d. injection of MDF. Rabbits were sacrificed 1 h later, injected sites were punched out and radioactivity determined. Ct/min/site was converted to the number of neutrophils/site. Neutrophil numbers are expressed as per million neutrophils per mL of blood. Each point represents the mean ± s.e. from 3–5 animals.

in doses of 300 μg 24 h and 1 h before inoculation with 100 μl of Evans blue dye and 5 μg of MDF. Plasma protein-bound dye leakage was measured 1 h later. Mice given drug and dye served as controls. The findings presented in Fig. 3 show that injection of indomethacin did not inhibit the alteration in dye-bound plasma leakage in mice (78 ± 11 μg/mouse) and it was similar to that in mice inoculated with MDF alone (82 ± 9 μg/mouse). The difference was insignificant ($P > 0.05$).

Effect of treatment with antihistaminic drugs on vascular permeability

Histamine receptor blockade experiments showed that plasma leakage was partially abrogated (48 to 50%) by treating the mice with 200 μg of Avil (H1 receptor blocker) or ranitidine (H2 receptor blocker) i.p. 30 min prior to MDF and dye injection, while co-injections of avil and ranitidine completely abrogated the plasma leakage (Fig. 3).

Effect of anti-MDF antiserum treatment

The response to MDF was inhibited by pretreatment of mice with anti-MDF antibody (dye–protein leakage 36 ± 6 μg/mouse) against MDF-induced increase in capillary permeability (dye–protein leakage 80 ± 13 μg/mouse). Normal rabbit serum had no effect on dye–protein leakage mediated by MDF (85 ± 15 μg/mouse).

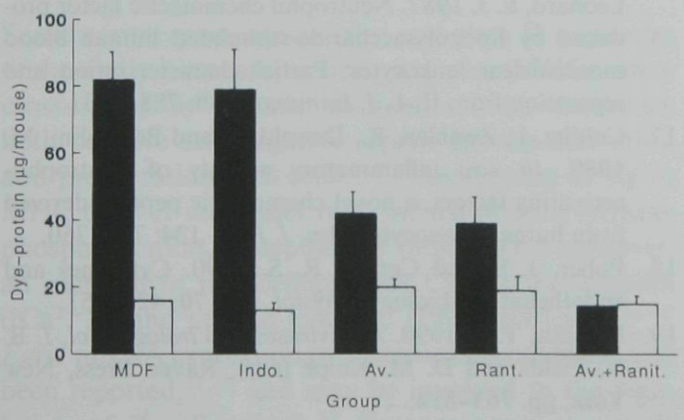

Fig. 3. Effect of pretreatment of mice with drugs (■): indomethacin (indo.), avil (Av.), ranitidine (rant.), and avil and ranitidine in combination on MDF-induced increase in capillary permeability. Control (□) mice received drugs and normal macrophage supernatant. Mice were injected with 300 μg of indomethacin i.p. 24 h and 1 h before 100 μL of Evans blue dye and MDF (5 μg) inoculation. Dye–protein leakage into the peritoneal cavity was measured 1 h later. Two hundred micrograms of avil or ranitidine separately or in combination was injected in groups of mice 30 min prior to dye and MDF inoculation, and peritoneal washings collected 1 h later were screened for dye–protein content spectrophotometrically. Each point represents the mean ± s.e. from 10 mice.

Discussion

After entry into the host, Japanese encephalitis virus replicates in a number of organs and generates a rapid inflammatory response with mononuclear and polymorphonuclear cell infiltration.[2,4] However, the exact mechanism involved in producing this inflammatory state is not understood.

The present study demonstrates that mice injected with JEV-stimulated mouse splenic macrophage-derived factor show increased capillary permeability, as demonstrated by the leakage of radiolabelled BSA or Evans blue dye into the extravascular space. The MDF-induced emigration of [^{51}Cr]-labelled neutrophils and plasma extravasation from the intravascular compartment to the site of injection peaked 1 h after inoculation. MDF is a low molecular weight neutrophil chemotactic peptide,[7] and seems to be similar to IL-8, which is a family of low molecular mass proteins (8–10 kDa) that is chemotactic for neutrophils[16] and can produce oedema.[17] Vascular leakage and neutrophil accumulation has also been attributed to the action of bacterial endotoxin or IL-1 and TNF, which bind to cell surface receptors, promote neutrophil attachment and subsequent emigration.[18] In the present study, the MDF preparation was free of endotoxin since *in vivo* inoculation of MDF with endotoxin inhibitor polymyxin B (10 μg/mL) did not inhibit the increase in vascular permeability (data not shown).

Several workers have reported that prostaglandins, bradykinins and histamine are potent endothelial vasodilators.[5] In our model, an involvement of prostaglandin in MDF-induced increases in vascular leakage was not observed as prostaglandin synthetase inhibitor had no effect, while drugs that block histamine receptors abrogated the MDF-induced capillary permeability.

It is intriguing that only 1 in 200 or 300 persons infected with JEV develops clinical encephalitis. Although JEV replicates in the vascular endothelial cells,[1] it remains unclear whether the exact mechanism of virus invasion in the central nervous system is via an olfactory or haematogenous route or whether disruption is caused by dual infection.[19] Recently, we have demonstrated leakage of radiolabelled erythrocytes, plasma proteins and accumulation of neutrophils into the brain tissue after i.v. injection of MDF in mice, suggesting the role of MDF in breaching the blood–

brain barrier.[3] It is likely that JEV, if in circulation during this period, gains access to the brain with consequent development of encephalitis. In the present study, anti-MDF antisera treatment protected the mice against the effects of MDF on the microvasculature. This shows that the adverse effects of MDF can be abrogated by such treatment. Experiments to show the homology of MDF with related inflammatory proteins are in progress.

We have previously reported transplacental transmission of JEV in humans and in mice.[20,21] It is likely that MDF plays an important role in the pathogenesis of fetal infection, as those viruses that cross the blood–brain barrier can also pass through the placental barrier.[22] Further work is in progress to unveil the pathophysiological role of MDF in Japanese encephalitis.

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