Clin. exp. Immunol. (1991) **86**, 299–303

Neutrophil chemotactic factor produced by Japanese encephalitis virus stimulated macrophages

N. KHANNA, M. AGNIHOTRI, A. MATHUR & U. C. CHATURVEDI
Postgraduate Department of Microbiology, K. G. Medical College, Lucknow, India

(Accepted for publication 22 May 1991)

SUMMARY

The mechanism of neutrophil leucocytosis in cases of Japanese encephalitis is not known. We here report that during Japanese encephalitis virus (JEV) infection in mice the splenic macrophages secrete a chemotactic factor that attracts the neutrophils. The peak activity of macrophage derived factor (MDF) was observed on day 7 following infection. The MDF acted in a dose-dependent manner. This chemoattractant was purified by low pressure liquid chromatography and gave a single band of 10 kD on silver stained polyacrylamide gel. The MDF was found to be heat resistant and sensitive to prolonged incubation with proteases.

Keywords Japanese encephalitis virus neutrophil chemotactic factor macrophage derived cytokine neutrophilia

INTRODUCTION

The chemotactic peptides released from lipopolysaccharide-stimulated human monocytes/macrophages (Wolpe *et al.*, 1988; Peveri *et al.*, 1988), T lymphocytes (Gregory *et al.*, 1988), B lymphocytes (Altman, Chassy & Mackler, 1975) and fibroblasts (Van Damme *et al.*, 1989) have been shown to attract neutrophils, produce granulocytosis and induce local skin inflammatory reaction. Some of the cytokines have been sequenced (Davatelis *et al.*, 1988; Matsushima *et al.*, 1988). A single peptide of 72 amino acids has been shown to be responsible for neutrophil-stimulating activity (reviewed by Westwick, Li & Camp, 1989). The chemotactic molecule has been recently renamed as IL-8 (Larsen *et al.*, 1989).

Leucopenia with lymphopenia is a frequent feature of most of the viral infections (deGruchy, 1976), while infection with Japanese encephalitis virus (JEV), a flavivirus (Shope, 1980) is characterized by polymorphonuclear leucocytosis with variable effect on different components of the peripheral blood leucocytes (Chaturvedi *et al.*, 1979). Our previous histopathological studies of mice showed initial accumulation of macrophages followed by neutrophils at the site of injury in the spleen (Mathur *et al.*, 1988). We have studied this phenomenon by priming mice with JEV and assaying the individual cell population for chemotactic activities. We present data demonstrating the production of a previously unrecognized neutrophil chemotactic cytokine secreted by the macrophages in spleen during JEV infection.

Correspondence: Professor Asha Mathur, Department of Microbiology, K. G. Medical College, Lucknow-226 003, India.

MATERIALS AND METHODS

Antigen and animals

JEV, strain 78668A, was used as infected mouse brain suspension. The infectivity titre of five times passed brain pool measured by intracerebral inoculation in infant mice was $10^{4 \cdot 1}$ 50% lethal dose (LD_{50}) per 25 μl. JEV given intraperitoneally produced no clinically evident disease, while intracerebral inoculation produced uniform sickness and 100% mortality by day 6 (Mathur, Arora & Chaturvedi, 1981). Inbred Swiss albino mice obtained from this Department were used throughout the study.

Preparation of macrophage derived factor (MDF)

The chemotactic factor was prepared from the spleen of JEV-infected mice. Briefly, the mice were given 0·3 ml of 10^2 LD_{50} of JEV intraperitoneally. The spleens were collected aseptically on day 7 post-infection (p.i.) and teased out in chilled HBSS. The cells (1×10^7/ml) were seeded in glass Petri dishes with MEM-Hepes (25 mM) for 1 h at 37°C. The non-adherent cells were removed and Petri dishes were thoroughly washed with phosphate-buffered saline (PBS). More than 90% of these cells were macrophages as judged by morphology and phagocytosis of latex particles (Mathur *et al.*, 1988). The adherent cells were cultured in saline for 24 h and the supernatant was tested for chemotactic activity.

Cell culture

The glass adherent cells were separated from normal mouse spleen in MEM-Hepes. The T and B lymphocyte-enriched populations were obtained by successive filtration of non-adherent cells through a nylon wool column by the modified technique of Julius, Simpson & Herzenberg (1973) as previously described (Mathur, Arora & Chaturvedi, 1983). The purity of T and B lymphocytes was checked by treating the cells with anti-Thyl.2 antisera or anti-mouse IgG antisera (New England Nuclear, Cambridge, MA) and complement. With this procedure pure populations of T and B lymphocytes (95% and 94% respectively) were obtained. The cells were washed three times with MEM. Total spleen cells, macrophages, T and B lymphocytes were cultured (5×10^6 cells/ml) in MEM-Hepes with antibiotics.

Purification of macrophage derived factor

The crude supernatant from splenic macrophages of JEV primed mice was concentrated by freeze drying in Speed Vac (Savant Instruments Inc., New York). The concentrated supernatant was subjected to low pressure liquid-chromatography (LPLC) using Sephacryl S-200 column (Pharmacia, Uppsala, Sweden) in 0·1 M PBS, pH 7·4, at flow rate of 100 drops/min and collected in 2·5 ml fractions. Absorbance was monitored at 280 nm as a parameter for protein concentration and the corresponding peaks were recorded (2210 recorder, LKB Instruments, Bromma, Sweden). The fractions were assayed for chemotactic activity. The active fractions were pooled and concentrated and applied again on Sephacryl S-200 column.

SDS–PAGE

The purity of chemotactic fraction was tested by SDS–PAGE in linear gradient (10–18%) polyacrylamide gels as described by Laemmli (1970). Silver staining of proteins in the gel was carried out according to the method of Merril *et al.* (1981). The following molecular weight standards were run in parallel: carbonic anhydrase (30 kD), trypsin inhibitor (21·5 kD), lysozyme (14·3 kD), aprotinin (6·5 kD).

Assay of neutrophil chemotaxis

Neutrophils were obtained from mice 6 h after i.p. inoculation of 1 ml of glycogen (0·1% in saline); more than 85% of the recovered cells were neutrophils. These were further enriched by gradient centrifugation (30 min, 400 *g*) on Ficoll–Hypaque. The cells in the pellet which consisted of 95% neutrophils were washed three times with HBSS and suspended (1×10^8 cells/ml) in HBSS. Neutrophil chemotaxis was assessed by a Boyden chamber assay (Pohajdak *et al.*, 1986). Briefly, the neutrophils ($1{\cdot}5 \times 10^6$ in 200 μl) in HBSS were placed in the top compartment and bottom chambers were filled with various chemoattractants or HBSS serving as negative control. N-formyl-methionyl-leucyl-pheny-lalanine (FMLP) (Sigma Chemical Co., St Louis, MO) at a concentration of 10^{-7} M was used as positive control. The chambers were incubated for 1 h at 37°C in a humidified 5% CO_2 atmosphere before the filters were removed, fixed in 70% isopropyl alcohol and stained with haematoxylin. The number of neutrophils migrated into the filter in high power fields (hpf, ×400) was counted. Each sample was tested in triplicate with neutrophil migration counted in five to seven fields and the mean migration ± s.e. was calculated.

Protein assay

Protein content was measured by the technique of Lowry *et al.* (1951).

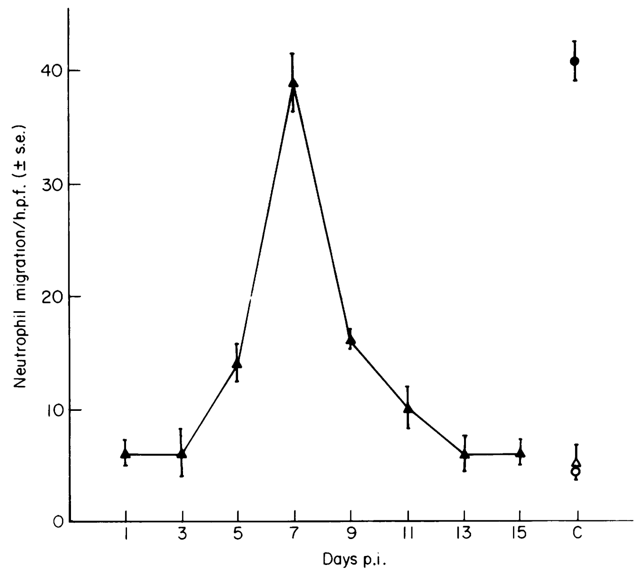

Fig. 1. Spleen cell neutrophil chemotactic activity at different periods of JEV-primed mice (▲), normal mice (△). FMLP (10^{-7}) (●) was used as positive control and MEM (○) as negative control. Each sample was tested in triplicate with neutrophil migration counted in five to seven high power field.

Protease treatment

Purified MDF was incubated at 37°C with and without trypsin (Sigma) or chymotrypsin (Sigma) (100 μg/ml) for 4 h and 18 h followed by addition of an excess of bovine serum albumin (BSA) (2 mg/ml) before the chemotactic activity of MDF was assayed.

RESULTS

Production of neutrophil chemotactic activity by spleen cells of JEV-primed mice

The ability of JEV-primed spleen cell supernatants to synthesize chemotactic cytokine was studied. Figure 1 shows that the maximum production of neutrophil chemotactic activity was obtained on day 7 following inoculation of 0·3 ml of 10 LD_{50} of JEV intraperitoneally (mean migration/hpf = 39 ± 1·9). Normal mouse spleen cell supernatant did not show detectable amounts of chemotactic activity (mean migration/hpf = 4 ± 1·5).

Identification of splenic cell population producing chemotactic activity

In order to delineate the cell type responsible for chemotactic activity *in vitro*, normal mouse splenic macrophages, T and B lymphocytes (5×10^6 cells/ml) were cultured and stimulated with 10^3 LD_{50} of JEV. The supernatants collected at 24 h, 48 h and 72 h were assayed for chemotactic activity. The chemotactic activity was produced by JEV-stimulated macrophages only, while T and B lymphocyte supernatants failed to attract neutrophils (Table 1). Control supernatants were found to be inactive.

In the present study the splenic macrophage culture supernatant of day 7 following JEV infection of mouse was used as chemotactic cytokine.

Table 1. Production of neutrophil chemotactic activity *in vitro*

JEV-stimulated culture supernatant	Neutrophil migration/hpf (hours)		
	24	48	72
Macrophage	18±1·6	30±3·2	22±0·8
T lymphocytes	3±0·5	4±0·7	2±0·5
B lymphocytes	5±0·7	6±0·3	2±0·5

The counts are presented after deducting the background values obtained with MEM. Number of neutrophils migrated/hpf with 10^{-7} M FMLP was 38±2·4. Values are presented as mean±s.e.

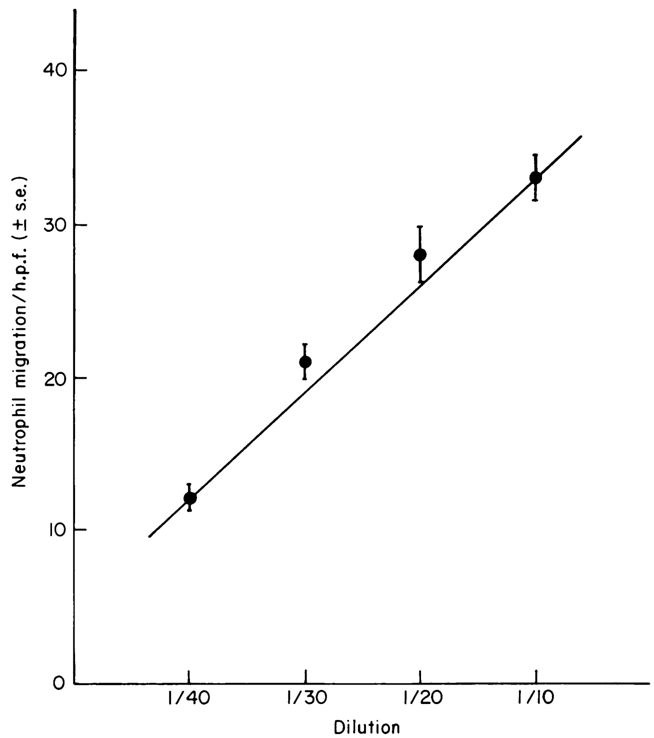

Fig. 2. Neutrophil chemotactic response at different dilutions of MDF. Each dilution was tested in triplicate with neutrophil migration counted in five to seven high power field.

Dose response of neutrophil chemotactic activity

Figure 2 shows that the chemotactic activity of MDF was dose-dependent. Progressive decrease in chemotactic activity was observed with increasing dilution of MDF.

Purification of macrophage-derived chemotactic activity

Crude supernatant of JEV-primed mouse splenic macrophage culture having neutrophil chemotactic activity was concentrated and applied to the Sephacryl S-200 column. Figure 3 shows the elution profile of different proteins present in the supernatant. Neutrophil chemotactic activity was recovered in peak 4 corresponding to fractions 23–26 and fractions 27 and 28. The active fractions were pooled, concentrated and applied on Sephacryl S-200 column again as a second purification step.

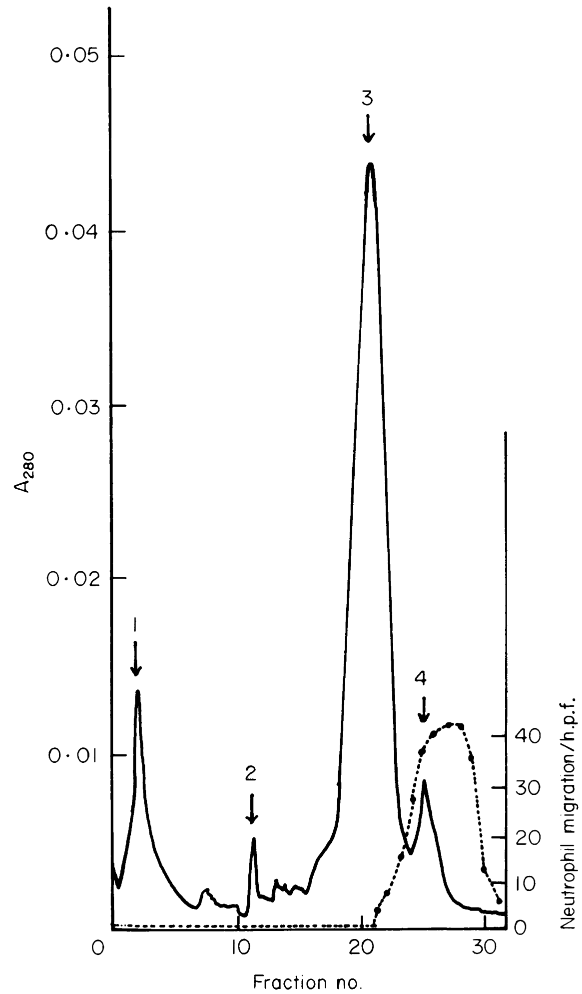

Fig. 3. Purification of macrophage-derived factor by low pressure liquid chromatography (——) and neutrophil chemotactic activity (- - - -).

The chemotactic activity was separated from peak 4 and was present in fractions 27 and 28 only. This was verified for purity and molecular weight determination by SDS–PAGE using 100 μg of purified MDF and showed a single band of 10 kD between lysozyme and aprotinin (Fig. 4).

Effect of JEV-antiserum treatment on MDF

The MDF was incubated with an equal volume of 1 : 10 diluted JEV-specific antiserum (supplied by the Director, National Institute of Virology, Pune, India) at 37°C for 1 h. Similarly treated normal mouse macrophage culture supernatant and MDF with diluent were used as controls. The chemotactic activity of antisera-treated MDF was assayed and compared with controls. The neutrophil chemotactic activity of MDF was not affected by JEV antiserum treatment (mean migration/hpf 33±1·9).

Characterization of MDF

Findings summarized in Table 2 show almost no loss in the chemotactic activity of MDF at 37°C, while heating to 100°C for 5 min resulted in a 34% reduction in activity. The MDF was frozen at −70°C without loss of activity. The pH of MDF was adjusted to 2 or 9 and incubated at 22°C for 3 h, followed by readjustment of pH to 7. Findings summarized in Table 2 show

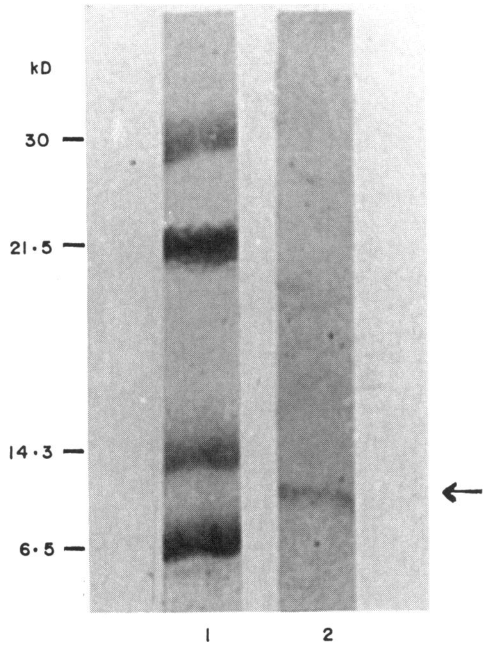

Fig. 4. SDS-PAGE of purified MDF. Molecular weight standards are applied in lane 1. The position of MDF in the gel is indicated by an arrow in lane 2.

Table 2. Physicochemical properties of MDF

Treatment	Neutrophil migration/ hpf (±s.e.)	Activity (% of control)
Trypsin (100 μg/ml)		
4 h, 37°C	31±3·4	100
Control	30±4·6	—
18 h, 37°C	1±0·4	3
Control	26±4·1	—
Chymotrypsin (100 μg/ml)		
4 h, 37°C	36±2·5	100
Control	35±5·0	—
18 h, 37°C	1·0±0·9	3
Control	27±1·1	—
Temperature		
37°C, 3 h	28±3·9	96
56°C, 1 h	28±2·8	96
100°C, 5 min	19±1·5	66
−70°C	30±2·0	100
Control (22°C)	29±0·5	—
pH 2·0		
22°C, 3 h	33±4·4	100
Control	32±3·6	—
pH 9·0		
22°C, 3 h	34±2·2	97
Control	35±3·0	—

Values are presented as mean of five duplicate experiments.

that the chemotactic activity of MDF was stable at acid and alkaline pH.

The chemotactic activity of the purified MDF was assayed after incubation with 100 μg/ml of trypsin or chymotrypsin for 4 h and 18 h. The results (Table 2) show no change in the chemotactic activity of MDF at 4 h while prolonged incubation with proteases resulted in complete loss of activity, indicating that MDF is a polypeptide.

DISCUSSION

Japanese encephalitis virus, a flavirirus (Shope, 1980) is the most important cause of encephalitis, with high mortality world over (Burke *et al.*, 1985; Mathur *et al.*, 1990). After haematogenous spread of the JEV, an early influx of macrophages with subsequent accumulation of neutrophils at the site of injury in humans (Johnson *et al.*, 1985) and in mice (Mathur *et al.*, 1988), polymorphonuclear leucocytosis (Chaturvedi *et al.*, 1979) and the presence of a large number of neutrophils in cerebrospinal fluid (Johnson, Intralawan & Puapanwatton, 1986) have been reported. Different cellular sources for soluble chemotactic factor production are leucocytes, fibroblasts, epidermal and endothelial cells (Westwick *et al.*, 1989). We have not come across any description of the release of neutrophil chemotactic factor during JEV infection.

Our findings demonstrated the production of a highly potent chemotactic factor by mouse splenic macrophages upon JEV challenge that attracts neutrophils. We have termed this compound macrophage derived factor (MDF). The induction of chemotaxis was a dose-dependent phenomenon with a peak chemotactic activity at day 7 p.i., indicating that its generation depends on the influx of mononuclear phagocytes into the affected tissue, as we have previously observed gradual increase in number of macrophages in the spleen from day 3 after JEV infection in mice followed by accumulation of neutrophils (Mathur *et al.*, 1988).

The MDF was found to be remarkably resistant to heat, acid and alkaline pH. On the basis of protease sensitivity it appears to be a polypeptide. MDF was purified by Sephacryl S-200 column chromatography and migrated as a single 10-kD protein band on SDS–PAGE. A low molecular weight neutrophil chemotactic protein has been reported after Con A or phytohaemagglutinin (PHA) stimulation of peripheral blood mononuclear cells (Van Damme *et al.*, 1988; Larsen *et al.*, 1989).

Mononuclear leucocytes secrete biologically active chemotactic cytokines in response to inflammatory stimuli (Westwick *et al.*, 1989). Neutrophil chemotactic peptides from lipopolysaccharide-stimulated monocytes have been identified and sequenced (Yoshimura *et al.*, 1987; Peveri *et al.*, 1988; Wolpe *et al.*, 1988). Over the past few years the chemotactic activity of granulocytes has been attributed to IL-1 (Dinarello & Mier, 1987). It has been suggested that IL-1 is not directly responsible for neutrophil chemotactic activity but can induce a chemotactic protein (Strieter *et al.*, 1988; Matsushima *et al.*, 1988). Recently, some groups have identified and sequenced the inflammatory neutrophil chemotactic protein, IL-8. The activity is due to a low mol. wt protein with molecular mass of about 10 kD (Yoshimura *et al.*, 1987). The mature chemotactic protein occurs as a 6–7 kD protein (Van Damme *et al.*, 1988) produced by various types of cells (Larsen *et al.*, 1989; Van Damme *et al.*, 1989) and is shown to be homologous with β-thromboglobulin and platelet factor-4 (Van Damme *et al.*, 1989). Identification of MDF by NH_2-terminal amino acid sequence analysis is in progress, in order to make a detailed comparison with other chemotactic cytokines.

JEV usually causes inapparent infection, but it can result in encephalitis in a few cases. The exact mechanism involved in initiation of brain infection is not known. We have recently observed leakage of protein-bound Evan's blue and ^{51}Cr-labelled erythrocytes into the extravascular space of brain tissue in MDF-injected mice (data not included) as the evidence of an increase in the capillary permeability resulting in breakdown of the blood–brain barrier. Although the exact pathophysiological relevance of MDF is yet to be established, the findings suggest that MDF released during JEV infection elicits a rapid inflammatory response *in vivo* with neutrophil accumulation at the site of injury.

REFERENCES

ALTMAN, L.C., CHASSY, B. & MACKLER, B.F. (1975) Physicochemical characterization of chemotactic lymphokines produced by human T and B lymphocytes. *J. Immunol.* **115,** 18.

BURKE, D.S., LORSOMRUDEE, W., LEAKE, C.J., HOKE, C.H., NISALAK, A., CHONGSWASDI, V. & LAORAKPONGSE, T. (1985) Fatal outcome in Japanese encephalitis. *Am. J. trop. Med. Hyg.* **34,** 1203.

CHATURVEDI, U.C., MATHUR, A., TANDON, P., NATU, S.M., RAJVANSHI, S. & TANDON, H.O. (1979) Variable effect on peripheral blood leucocytes during JE virus infection in man. *Clin. exp. Immunol.* **38,** 492.

DAVATELIS, G., OLSON, P.T., WOLPE, S.D., HERMSEN, K., LEUDKE, C., GALLEGOS, C., COIT, D., MEERYWEATHER, J. & CERAMI, A. (1988) Cloning and characterization of cDNA for murine macrophage inflammatory protein (MIP) a novel monokine with inflammatory and chemokinetic properties. *J. exp. Med.* **167,** 1939.

DEGRUCHY, G.C. (1976) *Clinical Haematology in Medical Practice.* 3rd edn., p. 359. Blackwell Scientific Publications, Oxford.

DINARELLO, C.A. & MIER, J.W. (1987) Lymphokines. *New Engl. J. Med.* **317,** 940.

GREGORY, N., YOUNG, J., SCHRODER, J.M., MROWITZ, U. & CHRISTOPHERS, E. (1988) Structure determination of a human lymphocyte derived neutrophil activating peptide (LYNAP). *Biochem. Biophys. Res. Commun.* **151,** 883.

JOHNSON, R.T., BURKE, D.S., ELWELL, M., LEAKE, C.J., NISALAK, A., HOKE, C.H. & LORSOMRUDEE, W. (1985) Japanese encephalitis. Immunocytochemical studies of viral antigen and inflammatory cells in fatal cases. *Ann. Neurol.* **18,** 567.

JOHNSON, R.T., INTRALAWAN, P. & PUAPANWATTON, S. (1986) Japanese encephalitis: Identification of inflammatory cells in cerebrospinal fluid. *Ann. Neurol.* **20,** 691.

JULIUS, M.H., SIMPSON, E. & HERZENBERG, L.R. (1973) A rapid method for the isolation of functional thymus derived murine lymphocytes. *Eur. J. Immunol.* **3,** 645.

LAEMMLI, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* **227,** 680.

LARSEN, C.G., ANDERSON, A.O., APPELLA, E., OPPENHEIM, J.J. & MATSUSHIMA, K. (1989) The neutrophil-activating protein (NAP-1) is also chemotactic for T lymphocytes. *Science,* **243,** 1464.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951) Protein measurements with the Folin phenol reagent. *J. biol. Chem.* **193,** 265.

MATHUR, A., ARORA, K.L. & CHATURVEDI, U.C. (1981) Congenital infection of mice with Japanese encephalitis virus. *Infect. Immun.* **34,** 26.

MATHUR, A., ARORA, K.L. & CHATURVEDI, U.C. (1983) Host defence mechanisms against Japanese encephalitis virus infection in mice. *J. gen. Virol.* **64,** 805.

MATHUR, A., BHARADWAJ, M., KULSHRESHTHA, R., RAWAT, S., JAIN, A. & CHATURVEDI, U.C. (1988) Immunopathological study of spleen during Japanese encephalitis virus infection in mice. *Brit. J. exp. Path.* **69,** 423.

MATHUR, A., KUMAR, R., SHARMA, S., KULSHRESHTHA, R., KUMAR, A. & CHATURVEDI, U.C. (1990) Rapid diagnosis of Japanese encephalitis by immunofluorescent examination of cerebrospinal fluid. *Ind. J. med. Res. (A)*, **91,** 1.

MATSUSHIMA, K., MORISHITA, K., YOSHIMURA, T., LAVU, S., KOBAYASHI, Y., LEW, W., APPELLA, E., KUNG, H.F., LEONARD, E.J. & OPPENHEIM, J.J. (1988) Molecular cloning of a human monocyte-derived neutrophil chemotactic factor (MDNCF) and the induction of MDNCF mRNA by interleukin 1 and tumour necrosis factor. *J. exp. Med.* **167,** 1883.

MERRIL, C.A., GOLDMAN, D., SEDMAN, S.A. & EBERT, M.M. (1981) Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. *Science,* **211,** 1437.

PEVERI, P., WALZ, A., DEWALD, B. & BAGGLIOLINI, M. (1988) A novel neutrophil-activating factor produced by human mononuclear phagocytes. *J. exp. Med.* **167,** 1547.

POHAJDAK, B., GOMEZ, J., ORR, F.W., KHALIL, N. & GREENBERG, A.H. (1986) Chemotaxis of large granular lymphocytes. *J. Immunol.* **136,** 278.

SHOPE, R. (1980) Medical significance of Togaviruses: An overview of disease in man and in domestic and wild vertebrate animals. In *Togaviruses* (ed. by R.W. Schlesinger) p. 47. Academic Press, New York.

STRIETER, R.M., KUNKEL, S.L., SHOWELL, H.J. & MARKS, R.M. (1988) Monokine-induced gene expression of a human endothelial cell-derived neutrophil chemotactic factor. *Biochem. Biophys. Res. Commun.* **156,** 1340.

VAN DAMME, J., BEEUMEN, J.V., OPDENAKKER, G. & BILLIAU, A. (1988) A novel NH_2-terminal sequence characterized human monokine possessing neutrophil chemotactic, skin reactive and granulocytosis-promoting activity. *J. exp. Med.* **167,** 1364.

VAN DAMME, J., DECOCK, B., CONING, R., LENAERTS, J.P., OPDENAKKER, G. & BILLIAU, A. (1989) The chemotactic activity for granulocytes produced by virally infected fibroblasts is identical to monocyte derived interleukin-8. *Eur. J. Immunol.* **19,** 1189.

WESTWICK, J., LI, S.W. & CAMP, R.D. (1989) Novel neutrophil stimulating peptides. *Immunol. Today,* **10,** 146.

WOLPE, S.D., DAVATELIS, G., SCHERRY, B., BEUTLER, B., HESSE, D.G., NGUYEN, H.T., MOLDAWER, L.L., NATHAN, C.F., LOWRY, S.F. & CERAMI, A. (1988) Macrophages secrete a novel heparin-binding protein with inflammatory and neutrophil chemokinetic properties. *J. exp. Med.* **167,** 570.

YOSHIMURA, T., MATSUSHIMA, K., TANAKA, S., ROBINSON, E.A., APPELLA, E., OPPENHEIM, J.J. & LEONARD, E.J. (1987) Purification of a human monocyte derived neutrophil chemotactic factor that has peptide sequence similarity to other host defence cytokines. *Proc. natl Acad. Sci. USA,* **84,** 9233.