

Effect of macrophage-derived factor on hypoferraemia induced by Japanese encephalitis virus in mice

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SUMMARY

Depression of serum iron following Japanese encephalitis virus (JEV) infection was observed in mice. The hypoferraemia was associated with the accumulation of iron in reticulo-endothelial cells in the spleen. Splenectomy (compared with sham-operation) prevented the depression in serum iron concentration after JEV infection. It also prevented the rise in levels of liver iron. The effect of JEV-stimulated, splenic macrophage-derived factor (MDF) was evaluated in causing hypoferraemia. MDF produced a rapid reduction in the serum iron levels with accumulation of iron in spleen. These observations suggest that MDF plays a key role in the regulation of iron metabolism during JEV infection.

Keywords Japanese encephalitis virus hypoferraemia cytokines macrophage-derived factor

INTRODUCTION

Several bacterial (Letendre & Holbein, 1983; Finkelstein, Sciortino & McIntosh, 1983) and viral (Pekarek *et al.*, 1970; Blumberg, 1986) infections result in alterations in iron metabolism. Lowering of serum iron during infection has been regarded as the host-mediated attempt to limit the infection by preventing utilization of iron by the invading organisms. Beisel (1976) proposed that lactoferrin released from leucocytes after exposure to bacterial endotoxin results in the formation of lactoferrin-iron complexes that are taken up by the liver and converted to haemosiderin, resulting in anaemia. The hypoferraemia is associated with inflammation because of a blockade in the release of iron from reticulo-endothelial cells, liver and intestinal mucosa (Cartwright & Lee, 1971); the exact mechanism involved is not fully understood.

Studies in our laboratory indicated that Japanese encephalitis virus (JEV) given intraperitoneally replicates in different organs (Mathur *et al.*, 1986, 1988), resulting in hypoferraemia due to the storage of iron in reticulo-endothelial cells in spleen (Mathur, Bharadwaj & Chaturvedi, 1990). Recently we have observed release of a neutrophil chemotactic factor by JEV-activated splenic macrophages (unpublished results). Recent studies have shown that interleukin-1 (IL-1) secreted by activated monocytes or macrophages is capable of inducing hypoferraemia (Dinarello, 1988). The aim of this work was to investigate the contribution of spleen in production of hypoferraemia during JEV infection.

We present data showing that JEV-stimulated, splenic macrophage-derived factor (MDF) plays a major role in regulating iron metabolism.

MATERIALS AND METHODS

Virus

JEV strain 78668A was used as an infected mouse brain suspension as described previously (Mathur, Arora & Chaturvedi, 1983). Infectivity titre of the virus in suckling mice was $10^{4.8}$ LD₅₀/0.025 ml. It produced 100% mortality by day 6 following intracerebral (i.c.) inoculation; i.p. inoculation produced no clinically evident disease.

Splenectomy

The abdominal cavity was opened by a left horizontal subcostal incision. The spleen was exteriorized, the pedicle ligated and spleen removed. Sham-operated mice were kept as controls. In these mice the abdominal cavity was opened, the spleen was exteriorized and then returned again to its place and the abdominal cavity was sutured. These mice were used for the experiments 6 weeks after the operation. Splenectomized and sham-operated mice given 0.3 ml of 100 LD₅₀ JEV intraperitoneally were killed in groups on alternate days after infection. Uninfected sham-operated or splenectomized mice served as control.

Iron determination

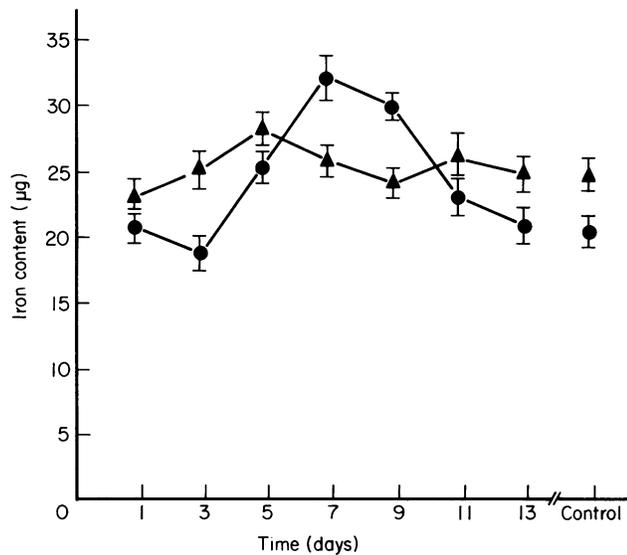
Inbred Swiss albino conventional male mice obtained from the mouse colony of our Department were used throughout the study. Blood was collected by cardiac puncture at varying

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Table 1. Mean values of serum iron, total iron binding capacity (TIBC) in JEV-infected sham-operated (Sham.) and splenectomized (Splx.) mice

Day post-infection	Serum iron ($\mu\text{g } \%$)		TIBC ($\mu\text{g } \%$)		Iron saturation (%)	
	Sham	Splx.	Sham	Splx.	Sham	Splx.
1	174 \pm 5	178 \pm 3	336 \pm 7	300 \pm 9	51.8	59.3
3	85 \pm 3	170 \pm 3	320 \pm 5	340 \pm 7	26.0	50.0
5	76 \pm 3	175 \pm 2	340 \pm 3	324 \pm 10	22.3	54.0
7	72 \pm 2	174 \pm 2	335 \pm 7	310 \pm 6	21.4	56.1
9	74 \pm 1	178 \pm 1.4	300 \pm 7	345 \pm 8	24.6	51.6
11	132 \pm 3	175 \pm 1.6	335 \pm 6	365 \pm 8	39.4	47.9
13	168 \pm 4	170 \pm 5	340 \pm 5	325 \pm 9	49.4	52.3
Control	176 \pm 4	175 \pm 3	335 \pm 6	340 \pm 5	54.3	51.4

Mean values of at least five to seven pooled serum samples. JEV, Japanese encephalitis virus.

**Fig. 1.** Total liver iron content in JEV-infected sham-operated (●) and splenectomized (▲) mice. Each point represents the mean iron content in μg per liver from five to six mice individually \pm s.d.

intervals from groups of JEV-infected and control mice. All the mice were bled in the morning at approximately the same time of the day. Serum iron and total iron binding capacity were determined colourimetrically according to the modified method of Beale, Bostrom & Taylor (1962) with bathophenanthroline sulphate as the colour reagent, as described elsewhere (Mathur *et al.*, 1990).

Estimation of tissue iron

The liver and spleen of JEV-infected and control mice were collected aseptically on alternate days. The tissues were individually dried, weighed and digested and iron content in spleen and liver were determined by atomic absorption spectrophotometry (Edward *et al.*, 1977) as described (Mathur *et al.*, 1990). Results are expressed in μg of iron per organ (iron content).

Table 2. Effect of macrophage-derived factor (MDF) and Japanese encephalitis virus (JEV) on serum iron in mice

Time post-infection (h)	Serum iron ($\mu\text{g } \%$)	
	MDF	JEV
6	182 \pm 11	170 \pm 3.4
12	165 \pm 5	173 \pm 4.1
24	76 \pm 2.3	175 \pm 7
48	80 \pm 3	143 \pm 5
72	125 \pm 9	87 \pm 2
Control	180 \pm 10	168 \pm 3

Values are expressed as mean of five to seven serum samples \pm s.e.m.

Perls' stain

Liver and spleen from groups of infected and control mice were removed. Tissue pieces were fixed in 10% neutral formalin. Paraffin blocks were made, sectioned and stained with Perls' Prussian blue stain for ferric iron.

Preparation of MDF

MDF was prepared from spleen cells of JEV-infected mice. Briefly, Swiss albino mice were primed with a single i.p. injection of 0.3 ml of 10^2 LD₅₀ of JEV. The spleens were harvested aseptically on day 7 post-infection (at the time of peak chemotactic activity) and gently teased until most of the cells were released from the capsule. The cells were washed three times with MEM and resuspended in 10 mM MEM-HEPES with antibiotics and plated at 1×10^7 cells/ml in glass Petri dishes for 2 h at 37°C in presence of 5% CO₂. After this, the medium with non-adherent cells was removed. The adherent cells were rinsed twice with phosphate-buffered saline (PBS) and were further cultured in saline for 24 h. The supernatant was collected and centrifuged at 400 g for 20 min at 4°C. The cell-free supernatant was assayed for chemotactic activity as described by Nelson, Quie & Simmons (1975). The protein content of the supernatant was measured by the technique of Lowry *et al.* (1951).

Purification of MDF

The supernatant from adherent spleen cell cultures was concentrated to 2 ml on a Diaflo membrane (Amicon, Danvers, MA). The concentrated material was added to 450 \times 16-mm Sephacryl S-200 column (Pharmacia, Uppsala, Sweden), and was eluted with 0.1 M PBS. The 2.5-ml fractions obtained were assayed for neutrophil chemotactic activity. The active fractions were pooled and concentrated 25 times on Diaflo membrane.

RESULTS

Assay of serum iron

The data presented in Table 1 show that in JEV-infected, sham-operated mice the serum iron level declined gradually and remained significantly lower than that of control mice up to day 9 post-infection, while in the splenectomized mice, following JEV infection serum iron levels remained unchanged throughout the study period and were similar to that in control splenectomized mice.

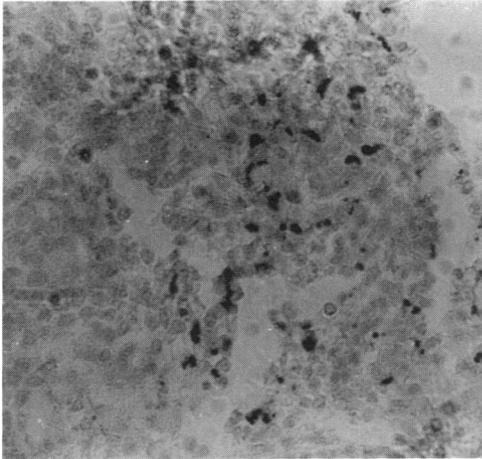


Fig. 2. A section of spleen after 1 day of inoculation of purified macrophage-derived factor showing iron deposition within splenic macrophages. Pearl's stain, magnification $\times 170$.

No significant difference was observed in the total iron binding capacity in JEV-infected, sham-operated and splenectomized mice (Table 1). The per cent of iron saturation declined in sham-operated mice from day 3 to 9 post-infection, and was more or less same in splenectomized mice.

Effect on liver iron

The findings summarized in Fig. 1 show the total iron content in liver of splenectomized and sham-operated mice on different days after JEV infection. In uninfected, sham-operated mice the mean iron content was $20.7 \pm 1.4 \mu\text{g}$. There was slight increase in the total iron content in sham-operated mice from day 5 to 9 after JEV infection, while in splenectomized mice no change in the iron content was observed after JEV infection and the values were similar to those of control mice.

The Prussian-blue-stained sections of liver from JEV-infected, sham-operated mice showed stainable iron in Kupffer cells on days 7 and 9 post-infection; no iron deposition was noted in splenectomized and control mice.

Effect on haemoglobin level

Transient decline was observed in the mean haemoglobin levels in the sham-operated mice on days 7 and 9 only (8.6 ± 1 to 9.4 g per cent) after JEV infection. In contrast, in JEV-infected splenectomized mice the mean values were similar to those of controls ($15 \pm 0.5\%$).

Effect on erythrocyte count

The erythrocyte counts in the splenectomized and sham-operated JEV-infected mice were similar to those of controls and ranged between 9 ± 1 and $10 \pm 2.1 \times 10^6$ cells/mm³.

Effect of MDF on iron metabolism

Since the above experiments showed the spleen plays a key role in iron metabolism, we investigated splenic MDF as a possible regulator of hypoferraemia. We observed that a purified preparation of MDF ($5.0 \mu\text{g}/\text{mouse}$ intravenously) caused significant depression (42%) in serum iron levels as measured 24 h and 48 h after inoculation (Table 2), while no changes were detected at 6 and 12 h of injection. In order to investigate further

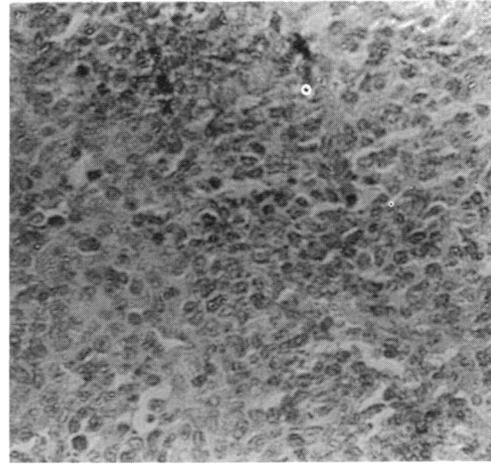


Fig. 3. A section of spleen of mice inoculated with normal mouse splenic macrophage culture supernatant. Pearl's stain, magnification $\times 170$.

the role of MDF in inducing alteration in iron metabolism, we studied the Pearl's-stained sections of the spleens of MDF-inoculated mice. The iron staining revealed increased iron deposition within splenic macrophages at 24 and 48 h, which gradually declined at 72 h after MDF injection (Fig. 2). No iron deposition was observed after injection of normal splenic macrophage culture supernatant (Fig. 3).

Effect of JEV antiserum treatment on MDF activity

A purified preparation of MDF was incubated with an equal volume of JEV-specific antiserum (supplied by the Director, National Institute of Virology, Pune, India) or with the diluent at 37°C for 1 h; then the chemotactic activity and the alteration in serum iron levels were assayed. The antisera-treated MDF depressed serum iron levels (mean at 24 h $72 \pm 3 \mu\text{g} \%$) which was similar to the $75 \pm 2 \mu\text{g} \%$ depression obtained by untreated MDF. The neutrophil chemotactic activity of treated and untreated MDF was approximately same.

DISCUSSION

We have reported earlier that mice infected with JEV showed decreased serum iron concentration (Mathur *et al.*, 1990). However, the mechanisms involved in initiating the hypoferraemic response in infections and other inflammatory states remained obscure. The present study was carried out to investigate the possible role of spleen and JEV-stimulated splenic MDF in producing hypoferraemia during JEV infection. Experiments with JEV-infected, sham-operated mice showed a marked decrease in serum iron levels up to day 9 after infection, with accumulation of iron in reticulo-endothelial cells as described previously in JEV-infected mice (Mathur *et al.*, 1990). We observed that splenectomy abrogates the JEV induced hypoferraemia. Interestingly, there was no accumulation of iron in the liver after JEV infection. These observations indicated that the spleen plays a key role in regulatory mechanism of iron metabolism during JEV infection.

JEV replicates *in vivo* in local tissue, leading to viraemia, and then disseminates in vascular tissues such as liver, spleen or thymus, with maximum virus titre on day 9 post-infection (Mathur *et al.*, 1986, 1988), along with leucocytosis showing

marked neutrophilia. These findings, together with the fall in the serum iron level described earlier (Mathur *et al.*, 1990) and in the present study, indicate a relation between neutrophilia and hypoferraemia. The extent of these changes depends on the severity of the inflammatory process.

Little is known of the mechanism of the hypoferraemic response during infection, although several regulators of iron metabolism have been described. Cartwright & Lee (1971) have suggested that hypoferraemia is produced by blockade of the return of iron from the reticulo-endothelial system. Letendre & Holbein (1983) found that during meningococcal infection in mice, hypoferraemia occurred by inhibition of transport of haemoglobin-iron from the cells to plasma transferrin pool. Dinarello (1988) has described that IL-1, also termed leucocytic endogenous mediator (LEM), released by activated monocytes, decreased the plasma iron concentration. LEM promotes the selective release of lactoferrin, an iron-binding protein (Klempner, Dinarello & Gallin, 1978), resulting in a decrease in serum iron (Kampschmidt, Pulliam & Upchurch, 1980). This is unlikely to occur during JEV infection, as large quantities of lactoferrin would be required to cause hypoferraemia, and we observed excessive accumulation of iron in macrophages and other iron-storing cells in the spleen.

We believe that during JEV infection the hypoferraemia was achieved by a MDF secreted by JEV-stimulated splenic macrophages only. The MDF has been characterized as a heat-resistant, low molecular weight protein (10 kD) appearing during the early phase (peak activity at day 7) of infection; it induces an early influx of neutrophils in addition to being chemotactic for neutrophils *in vitro* (unpublished results). This chemotactic cytokine we suggest is interleukin-8 (IL-8). Further identification by NH₂-terminal amino acid sequence is in progress. Recently it has been demonstrated that IL-8 exerts neutrophil chemotactic effects (van Damme *et al.*, 1989; Larsen *et al.*, 1989; Westwick, Li & Camp, 1989). Furthermore, Larsen *et al.* (1989) suggested that IL-1 induces production of IL-8.

We have shown that *i.v.* injection of purified MDF in mice depressed the serum iron concentration with large accumulation of stainable iron in spleen. The possibilities for the excessive accumulation of iron in spleen during JEV infection could be that either the MDF helps in promoting the storage of iron in the spleen or reticulo-endothelial cell processed iron release to plasma was impaired. It is not clear whether MDF acts directly upon macrophages or through other mediators. Our findings show that MDF is a unique JEV-induced macrophage factor with a variety of biological activities.

REFERENCES

- BEALE, R.N., BOSTROM, J.O. & TAYLOR, R.F. (1962) Improved rapid methods for the determination of iron content and binding capacity of serum. *J. clin. Pathol.* **15**, 156.
- BEISEL, W.R. (1976) Trace elements in infectious processes. *Med. Clinics North Am.* **60**, 831.
- BLUMBERG, B.S. (1986) Hepatitis B virus, iron and iron binding proteins. In *Viruses, Immunity and Immunodeficiency* (ed. by A. Szentivanyi & H. Friedman). p. 81. Plenum Press, New York.
- CARTWRIGHT, G.E. & LEE, G.R. (1971) The anaemia of chronic disorders. *Br. J. Haematol.* **21**, 147.
- DINARELLO, C.A. (1988) Biology of interleukin-1. *FASEB J.* **2**, 108.
- EDWARD, C.O., CARROLL, M., BARY, P. & CARTWRIGHT, G.E. (1977) Heredity hemochromatosis: diagnosis in siblings and children. *N. Engl. J. Med.* **297**, 7.
- FINKELSTEIN, R.A., SCIORTINO, C.V. & MCINTOSH, N.A. (1983) Role of iron in microbe host interactions. *Rev. infect. Dis.* **5**, S759.
- KLEMPNER, M.S., DINARELLO, C.A. & GALLIN, J.I. (1978) Human leucocytic pyrogen induces release of specific granule contents from human neutrophils. *J. Clin. Invest.* **61**, 1330.
- KAMPSCHMIDT, R.F., PULLIAM, L.A. & UPCHURCH, H.F. (1980) The activity of partially purified leucocytic endogenous mediator in endotoxin-resistant C3H/HeJ mice. *J. Lab. clin. Med.* **95**, 616.
- 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.
- LETENDRE, E. & HOLBEIN, B.E. (1983) Turnover in the transferrin iron pool during the hypoferric phase of experimental *Neisseria meningitidis* infection in mice. *Infect. Immun.* **39**, 50.
- 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. (1983) Host defence mechanisms against Japanese encephalitis virus infection in mice. *J. gen. Virol.* **64**, 805.
- MATHUR, A., BHARADWAJ, M. & CHATURVEDI, U.C. (1990) Alteration in iron levels in Japanese encephalitis virus infection. *J. exp. Pathol.* **71**, 307.
- MATHUR, A., ARORA, K.L., RAWAT, S. & CHATURVEDI, U.C. (1986) Persistence, latency and reactivation of Japanese encephalitis virus infection in mice. *J. gen. Virol.* **67**, 381.
- 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. *Br. J. exp. Pathol.* **69**, 423.
- NELSON, R.D., QUIE, P.G. & SIMMONS, R.L. (1975) Chemotaxis under agarose: a new and simple method for measuring chemotaxis and spontaneous migration of human polymorphonuclear leukocytes and monocytes. *J. Immunol.* **115**, 1650.
- PEKAREK, R.S., BURGHEN, G.A., BARTELLONI, P.J., CALIA, F.M., BOSTIAN, K.A. & BEISEL, W.R. (1970) The effect of live attenuated Venezuelan equine encephalomyelitis virus vaccine on serum iron, zinc and copper concentrations in man. *J. Lab. clin. Med.* **76**, 293.
- VAN DAMME, J., DECOCK, B., CONINGS, R., LENAERTS, J., 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.