# ROLE OF CALCIUM IN NEUTROPHIL ACTIVATION BY JAPANESE ENCEPHALITIS VIRUS-INDUCED MACROPHAGE DERIVED FACTOR

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**Summary.** – The role of cytosolic $Ca^{2+}$ concentration in neutrophils stimulated with macrophage derived neutrophil chemotactic factor (MDF) produced following Japanese encephalitis virus (JEV) infection in mice was correlated with cell functions. MDF-induced $Ca^{2+}$ influx from extracellular milieu and release from intracellular store resulted in rise of cytosolic $Ca^{2+}$ in a dose-dependent manner and was independent of protein kinase C. Macrophages and B cells did not show cytosolic $Ca^{2+}$ changes while T lymphocytes showed slight rise when stimulated with MDF. Neutrophil chemotaxis in the absence of $Ca^{2+}$ was slightly different from that in presence of $Ca^{2+}$. Pretreatment of neutrophils with 3,4,5-trimethoxy-benzoic acid-8-(diethylamine)-octylester (TMB-8) inhibited the chemotaxis. It was observed that superoxide production and degranulation by neutrophils after stimulation with MDF was not dependent on the presence of extracellular calcium, but stripping of intracellular calcium resulted in abrogation of neutrophil activation. Thus, mobilization of intracellular calcium seems to be necessary for neutrophil activation by MDF.

**Key words**: macrophage derived chemotactic factor; neutrophil activation; Japanese encephalitis virus; calcium

## Introduction

In JEV infection the virus replicates in spleen (Mathur *et al.*, 1988) and other organs (Monath, 1990), and provides a strong stimulus for accumulation of macrophages which subsequently produce a neutrophil chemotactic factor. MDF is characterized as a 10 K polypeptide (Khanna *et al.*, 1991). It attracts neutrophils *in vitro*, induces neutrophil leucocytosis (Mathur *et al.*, 1992) and causes infiltration of neutrophils at the site of injection (Khanna *et al.*, 1994). MDF displays a variety of *in vivo* biological effects, e.g. breakdown of blood brain barrier (Mathur *et al.*, 1992), enhancement of capillary permeability (Khanna *et al.*, 1994), regulation of iron metabolism (Bharadwaj *et al.*, 1991) and activation of neutrophils (Khanna *et al.*, 1993).

Neutrophil functions can be mediated by mechanisms which are dependent or independent on $Ca^{2+}$. Several studies have shown that extracellular $Ca^{2+}$ is essential for optimal neutrophil chemotaxis, although similar migration of cells in the absence of external $Ca^{2+}$ has been demonstrated (Omann *et al.*, 1987). It has been documented that intracellular $Ca^{2+}$ plays a vital role in neutrophil migration as intracellular $Ca^{2+}$ chelators inhibit the neutrophil migration (Marasco *et al.*, 1984). Furthermore, various chemotactic stimuli can initiate neutrophil activation but vary in their efficacy in producing calcium permeability changes and activation responses such as superoxide generation and enzyme secretion (Korchak *et al.*, 1984). The production of reactive oxygen metabolites has been implicated in host defense against infections. The precise mechanism of neutrophil activation during JEV infection is not known and thus present study was undertaken to investigate the role of $Ca^{2+}$ in activation of target cells by MDF.

## Materials and Methods

*Virus.* JEV strain 78668A was used in the form of infected mouse brain suspension (Mathur *et al.*, 1983). The infectivity titer of virus in suckling mice was $10^{4.8}$ $LD_{50}$/0.025 ml. It produced

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100 % mortality within 6 days after intracerebral (ic) inoculation, while intraperitoneal (ip) inoculation produced no clinically evident disease.

*Preparation of MDF.* MDF secreted by spleen cells of JEV-infected mice showed peak chemotactic activity on day 7 p.i. and was prepared as described earlier (Khanna *et al.*, 1991). Briefly, mice were inoculated ip with 0.3 ml of 10 % JEV-infected mouse brain suspension. The spleens were collected aseptically on day 7 p.i., a single cell suspension ($1 \times 10^7$ cells/ml) in Minimal Eagle's Medium buffered with Hepes (MEM-HEPES) was seeded in glass Petri dishes and incubated for 2 hrs at 37 °C in 5 % $CO_2$ atmosphere. The adherent cells were washed thrice with PBS and cultured in saline for 24 hrs. The supernatant was collected, centrifuged at 4000 rpm for 15 mins and assayed for neutrophil chemotactic activity by Boyden chamber technique as described earlier (Khanna *et al.*, 1991). The supernatant was concentrated by freeze-drying in Speed Vac (Savant Instruments Inc., USA). MDF was purified by low pressure liquid chromatography (LPLC; Pharmacia) using Sephacryl S-200 column and eluted with 0.1 mol/l PBS. The protein peak with chemotactic activity was collected, concentrated and stored at –70 °C. 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 neutrophil chemotaxis* was done using a modification of the Boyden chamber technique as described earlier by Khanna *et al.* (1991) using 5μ pore size nitrocellulose filter. $10^{-7}$ mol/l N-formyl-methionyl-leucyl-phenylalanine (FMLP) was used as a positive control. The number of neutrophils which has migrated into the filter at the end of 1 hr incubation was counted in five to seven randomly selected fields (magnification 400 ×).

*Isolation of neutrophils.* Neutrophils were isolated from the peritoneal cavity of mice 4 hrs after ip inoculation of 1 ml of 0.1 % glycogen in saline. The exudate was collected by flushing the peritoneal cavity with MEM-HEPES supplemented with 0.5 unit/ml of heparin. Neutrophils were further enriched on Hypaque/Ficoll gradient (Khanna *et al.*, 1991). 97 % purified neutrophils were suspended in MEM after washing three times. Calcium was absent unless indicated.

*Preparation of splenic enriched cell population.* The enrichment of spleen cell subpopulations was carried out as described earlier (Mathur *et al.*, 1988). Briefly, single cell suspension of the spleen was seeded into glass Petri dishes and incubated at 37 °C for 90 mins in 5 % $CO_2$ atmosphere. Macrophage enriched cell population was obtained by collecting the cells adherent to glass. About 97 % of these cells phagocytosed latex particles and were considered as macrophages. Enriched subpopulation of T and B lymphocytes were prepared by filtration of glass non-adherent spleen cells through nylon wool column as described earlier (Mathur *et al.*, 1988).

*Assay of $^{45}Ca$ uptake* by neutrophils was done by the technique of Birx *et al.* (1984). Briefly, neutrophils ($1 \times 10^7$ cells/ml) were preincubated for 5 mins at 37 °C and MDF (5 μg/ml) was added. After 2 mins 0.1 ml of 0.2 μCi of $^{45}Ca$ was added to the cells and the reaction was stopped 30 mins later in ice bath. The cells were washed thrice with PBS containing 1.8 mmol/l $CaCl_2$ and harvested onto fibre glass filter. The radioactivity was measured in LKB-Wallac Beta Counter. The cpm in control and test groups were calculated by subtracting background cpm. The experiments were performed in triplicate and their results were presented as means ± SE.

*Superoxide assay.* The generation of $O_2^-$ by MDF-treated neutrophils was measured as superoxide dismutase inhibitable cytochrome C reduction (Johnston *et al.*, 1975).

*Exocytosis.* The release of granule-associated enzyme from cytochalasin B-treated and MDF-stimulated neutrophils was measured. Activity of beta-glucuronidase, an azurophil granule marker was determined using phenolphthalein-beta-glucuronide as substrate (Holligen *et al.*, 1952). Vitamin $B_{12}$-binding protein content was measured using $^{57}Co$-cyanocobalamin by the method of Dewald and Baggiolini (1986). Total enzyme activities were determined simultaneously in duplicate.

*Reagents.* Staurosporine (Sigma) was dissolved in dimethyl sulphoxide and was kindly provided by Dr. R. Martin, Lansing, USA. Cytochalasin B and FMLP (Sigma) were dissolved in dimethyl sulphoxide and diluted in PBS before use. $^{45}CaCl_2$ (specific activity of 105 μCi/g) and $^{57}Co$-cyanocobalamin (specific activity 1 μCi/μg) were obtained from Bhabha Atomic Research Centre, Bombay, India. EGTA, TMB-8 and cytochrome C (Sigma) were used.

## Results

### *Calcium permeability changes in neutrophils in response to MDF stimulation*

The $^{45}Ca$ uptake by neutrophils suspended in medium containing 1 mmol/l $CaCl_2$ or in calcium-free medium with 2 mmol/l EGTA in response to MDF was measured for 2 mins. The results (Fig. 1) show that the elimination of external $Ca^{2+}$ reduced, but did not completely inhibit the MDF-induced rise in cytosolic $Ca^{2+}$. It seems that the rise in intracytosolic calcium is only partially dependent on the presence of extracellular $Ca^{2+}$.

To investigate the contribution of the presence of intracellular calcium upon $^{45}Ca$ uptake, the neutrophils were exposed to 500 μmol/l TMB-8, an inhibitor of intracellular calcium mobilization (Korchak *et al.*, 1984), prior to MDF (prepared in $Ca^{2+}$ free medium) stimulation. It was observed that the TMB-8 pretreatment significantly inhibited calcium uptake by neutrophils (Fig. 1). This indicates the requirement of intracellular $Ca^{2+}$ for amplifying the calcium uptake. The $^{45}Ca$ uptake by MDF-stimulated neutrophils was higher than that with FMLP in all experimental conditions.

Fig. 2 shows a concentration dependent rise in $^{45}Ca$ uptake by neutrophils. Optimal $^{45}Ca$ uptake was observed with 5μg/ml MDF. At higher concentrations of MDF no further increase in response was observed while 1 μg/ml MDF did show detectable amount of stimulatory effect.

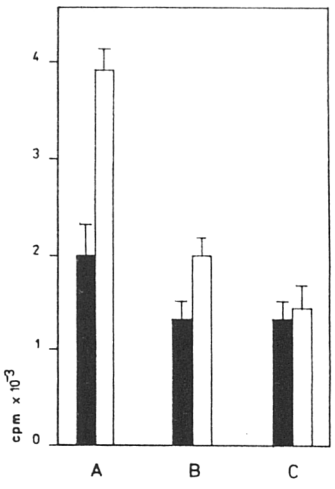


**Fig. 1**
**$^{45}$Ca influx in neutrophils in presence and absence of calcium**
MDF prepared in calcium containing medium (A); MDF prepared in calcium-free medium and cells suspended in calcium-free medium (B); MDF prepared in calcium-free medium and cells pretreated with TMB-8 (C). MDF-treated cells (black columns), control cells (empty columns).
Each column represents the mean ± S.E. of triplicate experiments.

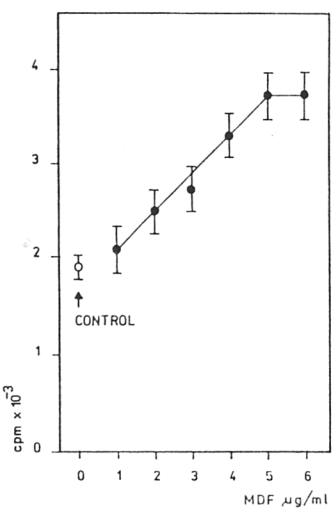


**Fig. 2**
**Effect of MDF on calcium permeability in neutrophils**
Each point represents the mean ± S.E. of triplicate experiments.

### *Effect of staurosporine on $^{45}$Ca uptake in neutrophils*

The effect of protein kinase C inhibitor, staurosporine on MDF-induced $^{45}$Ca uptake in neutrophils was investigated. The $^{45}$Ca uptake of neutrophils ($1 \times 10^7$ cells/ml) pretreated with different concentrations of staurosporine for 2 mins and stimulated with 5 µg/ml MDF or $10^{-7}$ mol/l FMLP was measured. No significant alteration in free cytosolic calcium after treatment with staurosporine was observed (Table 1), thereby showing that changes in calcium permeability in neutrophils upon MDF stimulation were independent of protein kinase C.

**Table 1. Effect of staurosporine on $^{45}$Ca uptake in neutrophils**

| Staurosporine concentration (nmol/l) | $^{45}$Ca uptake (cpm ± S.E.) | |
|---|---|---|
| | MDF | FMLP |
| 0 (control) | 2931 ± 286 | 2591 ± 229 |
| 10 | 2879 ± 282 | 2568 ± 237 |
| 20 | 2847 ± 271 | 2557 ± 229 |
| 40 | 2829 ± 264 | 2879 ± 237 |

Neutrophils ($1 \times 10^7$/ml) were incubated with different concentrations of staurosporine for 2 mins and stimulated with either 5µg/ml MDF or $10^{-7}$mol/l FMLP.

**Table 2. Calcium dependence of neutrophil chemotaxis**

| Stimulus | Neutrophil migration[c] |
|---|---|
| MDF+calcium[a] | 35 ± 4.1 |
| MDF+EGTA[b] | 32 ± 3.7 |
| TMB-8+MDF | 10 ± 2.4 |
| FMLP | 37 ± 2.1 |
| PBS | 4 ± 0.8 |

[a]MDF prepared in calcium containing medium, cells suspended in calcium-containing medium.
[b]MDF prepared in calcium free medium, cells suspended in mediumwith EGTA.
[c]Values are presented as means ± S.E.

### Calcium dependence of neutrophil chemotaxie

This experiment was performed in an attempt to establish whether or not $Ca^{2+}$ is necessary for neutrophil chemotaxis by MDF. MDF prepared in calcium-free medium was assayed for chemotaxis of neutrophils suspended in PBS with 2 mmol/l EGTA. Control MDF was prepared in the presence of $Ca^{2+}$ and chemotactic activity was assayed on cells suspended in calcium containing medium. The findings (Table 2) show that neutrophil chemotactic activity was not significantly affected when $Ca^{2+}$ was absent. Thus, it indicates that the requirement of extracellular $Ca^{2+}$ is not critical for MDF-induced neutrophil chemotaxis.

In another experiment, the effect of TMB-8, an inhibitor of intracellular calcium mobolization was determined. Neutrophils ($2.5 \times 10^6$/200 µl) were treated with $5 \times 10^{-4}$ mol/l TMB-8 before assaying for chemotaxis. The results showed that the pretreatment of neutrophils with TMB-8 inhibited the MDF-induced migration of neutrophils (Table 2). This

shows that the chemotaxis is partially dependent on the mobilization of intracellular calcium in neutrophils.

*Effect of calcium on neutrophil function*

We have previously demonstrated MDF-induced increased oxidative burst and enzyme release (Khanna *et al.*, 1993). In the present experiments we investigated the role of $Ca^{2+}$ in neutrophil activation. The neutrophils ($2.5 \times 10^6$/ml) pretreated with 5 μg/ml cytochalasin B were incubated either in the presence of 2 mmol/l EGTA or 1 mmol/l $CaCl_2$ and stimulated with 5 μg/ml MDF. Cytochrome C reduction was measured spectrophotometrically. The data presented in Fig. 3 show that MDF triggered $O_2^-$ generation response efficiently in calcium-free medium and was of similar magnitude as in calcium containing medium. FMLP stimulation also resulted in $O_2^-$ generation in both the conditions. This suggests that MDF-induced respiratory burst was independent of exogenous calcium.

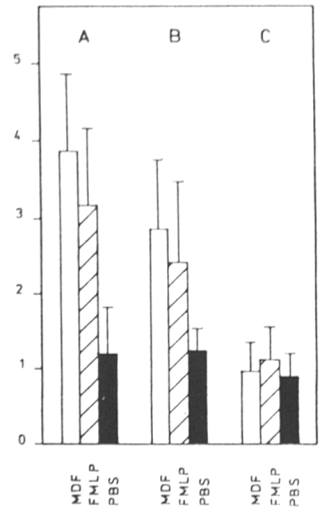


**Fig. 3**
**Effect of calcium on MDF- and FMLP-induced $O_2^-$ production**
Cells suspended in calcium containing medium (A) or medium with EGTA (B). Cells pretreated with TMB-8 (C). The $O_2^-$ production induced by MDF or FMLP. Control "induced" by PBS. Each column represents the mean ± S.E. of five experiments. Ordinate: nmoles of cytochrome C reduced/$10^6$ cells.

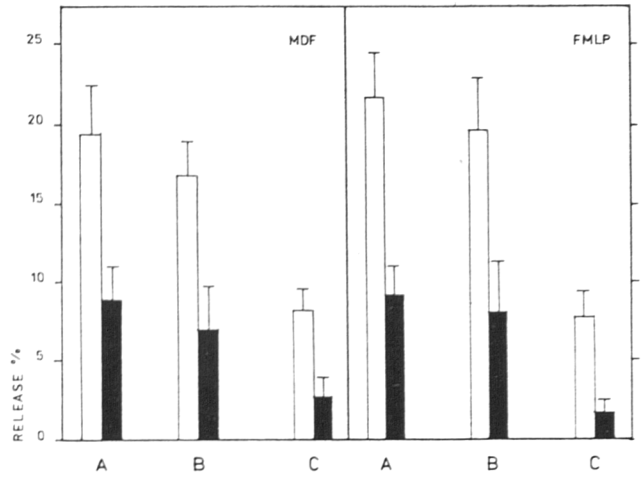


**Fig. 4**
**Effect of calcium on MDF- and FMLP-induced vitamin $B_{12}$ binding protein and beta-glucuronidase release**
Cells suspended in calcium containing medium (A), or medium with EGTA (B). Cells pretreated with TMB-8 (C). Vitamin $B_{12}$ binding protein release in % (empty columns), beta-glucuronidase release in % (black columns). Each column represents the mean ± S.E. of five experiments.

**Table 3. Assay of $^{45}Ca$ uptake in JEV-stimulated neutrophils on different days p.i.**

| Days p.i. | cpm | Alteration (%) |
|---|---|---|
| 5 | 2728 ± 214 | 63 |
| 7 | 3112 ± 298 | 97 |
| 9 | 2588 ± 242 | 55 |
| Control | 1688 ± 149 | – |

Peritoneal neutrophils of JEV-infected mice collected on different days p.i. were incubated with 0.2μCi/0.1ml of $^{45}Ca$. Radioactivity (cpm) was assayed and alteration (%) in intracellular calcium was calculated by the following formula:

$$\text{Alteration (\%)} = 100 \times \frac{(\text{cpm of MDF–stimulated cells}) - (\text{background cpm})}{(\text{cpm of control–stimulated cells}) - (\text{background cpm})} - 100$$

Control mice received normal mouse brain suspension. Data are presented as means ± S.E. of five experiments.

In another set of experiments the role of $Ca^{2+}$ on degranulation was studied. Cytochalasin B-treated neutrophils ($1 \times 10^8$/900 μl) suspended in calcium-free or calcium containing medium were stimulated with 5 μg/ml MDF and the release of vitamin $B_{12}$ binding protein and beta-glucuronidase were measured. The results showed that the MDF-induced increase in granule enzyme secretion takes place both in presence or absence of extracellular calcium (Fig. 4).

Since the above experiments showed that the MDF-induced neutrophil activation was not dependent on extracellular calcium, the MDF effect on $O_2^-$ production and enzyme release in TMB-8-treated neutrophils were examined. Fig. 3 and 4 show negligible $O_2^-$ production and granule enzyme release after TMB-8 treatment of cells indicating thereby that intracellular calcium mobilization is obligatory for neutrophil activation by MDF.

*$^{45}Ca$ uptake elicited by macrophages and lymphocytes*

The MDF-initiated $^{45}Ca$ uptake in enriched subpopulations of macrophages, T and B lymphocytes were determined and compared with FMLP. No rise in free cytosolic calcium concentration was observed in macrophages and B lymphocytes upon MDF stimulation, whereas T lymphocytes showed a small rise of 15 % in $^{45}Ca$ uptake.

*Effect of JEV on neutrophil activation*

Since the above experiments showed that MDF, secreted by splenic macrophages with peak activity on day 7 following ip inoculation of JEV, induced rise in cytosolic free $Ca^{2+}$, we investigated the ability of JEV to induce $^{45}Ca$ uptake in neutrophils *in vivo* and correlated it with cell functions on day 5,7 and 9 p.i. The findings (Table 3) show maximum increase in $^{45}Ca$ uptake in neutrophils on day 7. Cytochrome C reduction in neutrophils ($2 \times 10^6$ cells/ml) collected on different days from JEV-infected or control mice was assayed. JEV-induced exocytosis was assayed by measuring the release of vitamin $B_{12}$ binding protein and beta-glucuronidase from neutrophils. Table 4 shows that JEV efficiently stimulated exocytosis and $O_2^-$ generation.

**Table 4. JEV induced superoxide production and exocytosis on different days p.i.**

| Days p.i. | Cytochrome C reduction (nmoles/$10^6$ cells/2mins) | Granule enzyme release (%) | |
|---|---|---|---|
| | | Vitamin B12 | beta-glucuronidase |
| 5 | 2.8 ± 0.96 | 14.2 ± 2.4 | 7.6 ± 1.6 |
| 7 | 3.4 ± 1.2 | 19.0 ± 3.1 | 10.1± 1.9 |
| 9 | 3.0 ± 0.80 | 17.7 ± 2.9 | 8.1± 1.79 |
| Control | 1.08 ± 0.29 | 5.3 ± 0.82 | 3.8 ± 0.45 |

Neutrophils were collected from mice on different days after ip inoculation of 0.3 ml of 10 $LD_{50}$ of JEV. Control mice were inoculated with normal mouse brain suspension. Values are presented as means ± S.E. of five experiments.

## Discussion

MDF produced during JEV infection, is a chemotactic (Khanna *et al.*, 1991) and activating (Khanna *et al.*, 1983), low $M_r$ peptide for neutrophils. The present study demonstrates that MDF initiated a rise in free cytosolic $Ca^{2+}$, which was partially dependent on the presence of extracellular $Ca^{2+}$ as calcium influx studies using $CaCl_2$ in medium showed increase in cytosolic $Ca^{2+}$. However, $Ca^{2+}$ antagonist TMB-8 blocked the MDF-induced mobilization of intracellular calcium. Chemoattractants such as FMLP (Pozzan *et al.*, 1983; Korchak *et al.*, 1984) neutrophil activating factor/IL-8 (Thelen *et al.*, 1988) or platelet activating factor (von Tscharner *et al.*, 1986), stimulate and induce a rapid increase in free cytosolic $Ca^{2+}$ in neutrophils.

It was observed that the presence of extracellular $Ca^{2+}$ in the milieu was not necessary for neutrophil chemotactic activity of MDF, while the inhibition of $Ca^{2+}$ mobilization from intracellular organelles inhibited the chemotaxis. Thus, neutrophil migration by MDF is dependent on intracellular $Ca^{2+}$. Some investigators have shown that the chemotactic activity is extracellular calcium-dependent while others demonstrated neutrophil migration in the absence of calcium. The neutrophil chemotactic activity of bacterial peptide or C5a (Wilkinson, 1975) is extracellular calcium-dependent while FMLP does not require extracellular calcium. Elferink *et al.* (1992) have found that intracellular $Ca^{2+}$ only enhances the chemotaxis.

Attempts to assess the effect of $Ca^{2+}$ mobilization after stimulation with MDF on superoxide generation and exocytosis revealed that these responses require intracellular $Ca^{2+}$, since EGTA in medium did not abolish these responses, while TMB-8 (intracellular $Ca^{2+}$ mobilization inhibitor) abrogated the responses. Pozzan *et al.* (1983) have shown that stimulation with ionomycin is able to increase intracellular $Ca^{2+}$ but it is unable to elicit degranulation or superoxide generation, while FMLP causes rise in intracellular $Ca^{2+}$ with significant increase in granule enzyme release and superoxide formation. Incomplete secretogens ConA and PMA provoke the release of specific but not azurophil granules (Korchak *et al.*, 1984), but are effective stimuli for $^{45}Ca$ uptake indicating that azurophil granule release and $^{45}Ca$ uptake are not inevitably linked.

Previous studies have shown that the mobilization of intracellular $Ca^{2+}$ is necessary for neutrophil activation. The present study shows that the rise in cytosolic $Ca^{2+}$ in neutrophils induced by MDF was identical before and after treatment with staurosporine, while the respiratory burst was inhibited, indicating thereby that neutrophil activation by MDF is dependent on protein kinase C (Khanna *et al.*, 1993). Di Virgilio *et al.* (1985) and Robinson *et al.* (1984) have shown that diacylglycerol and low doses of tumour promotors stimulate protein kinase C synergistically and/or sequentially with $Ca^{2+}$, and high doses of calcium can activate protein kinase C independently of $Ca^{2+}$. Thus, present study demonstrates unambiguously the requirement of intracellular $Ca^{2+}$ mobilization in receptor-mediated cell stimulation.

The results of the present study show that the rise in intracytosolic $Ca^{2+}$ in neutrophils occurs on day 7 following JEV infection, coinciding with production of neutrophil chemotactic factor in spleen. It is possible that MDF helps in activation of cells. It has been demonstrated that the rise

in intracellular $Ca^{2+}$ is required for the initiation of neutrophil activation by chemoattractants, which may act directly or via calcium/calmodulin-dependent protein kinases (Omann *et al.*, 1987). Thus, MDF acts as a selective activator of neutrophils which is directly associated with increase in intracellular calcium.

Our previous work has shown hypercellular spleens with lymphoproliferative changes and infiltration of macrophages and neutrophils following JEV infection in mice (Mathur *et al.*, 1988). The present *in vivo* experiments indicate neutrophil activation with production of $O_2^-$ and secretion of lysosomal enzymes. Macrophages and neutrophils play vital roles in host defense against some viral infections (Daher *et al.*, 1986; Tsuru *et al.*, 1987). It remains to be investigated whether activated neutrophils can provide protection in JEV infection or not.

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