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Virus - Host Interplay During Japanese Encephalitis Virus Infection

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ABSTRACT

Our studies of an extensive epidemic of encephalitis at Gorakhpur (U.P.) in 1978, involving large segment of population with high mortality, revealed that the epidemic was due to Japanese encephalitis virus infection. Etiological diagnosis was based on virus isolation from brain of a fatal case and by serology. Large number of cases had polymorphonuclear leucocytosis. Our further studies had demonstrated the association of production of antigen-specific macrophage derived chemotactic factor (MDF) with polymorphonuclear leucocytosis during JEV infection in patients and in experimental mouse model. MDF is a 10 KD protein with plethora of biological effects. Immunological potentialities of purified MDF e.g. ability to activate neutrophils, regulate granulocytosis, increase capillary permeability with leakage of plasma protein, erythrocytes and cellular infiltrate in brain, lowering of serum iron levels with accumulation of iron in the spleen, degrade virus via triggering respiratory burst, generation of toxic oxygen radicals and production of nitric oxide has been discussed and is postulated that this may be one of the important mechanisms of natural immunity in controlling the initial stage of infection.

Our in vitro studies suggest the development of good humoral as well as cell mediated immune response to JEV experimentally in mice. Following infection, IgM antibodies, having neutralization and haemagglutinating activities appear first followed by IgG antibodies. JEV also triggers development of cell-mediated immunity (CMI) simultaneously. The leucocyte migration inhibition test was taken as an index of CMI. Passive transfer of JEV-specific antibodies or immune spleen cells provided short-term protection against challenge infection with JEV. Primary infection in few is followed by establishment of latent infection both experimentally in mice and in human cases. Latency is associated with depressed cell-mediated immune response. We demonstrated for the first time human transplacental transmission of JEV and developed the mouse model. Our studies had revealed an interesting observation of transmission of virus in consecutive pregnancies.

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Japanese encephalitis (JE)

JE is the principal mosquito-borne viral encephalitis in Asia. It is caused by Japanese encephalitis virus (JEV), a member of the family Flaviviridae. It is one of the most important examples of zoonotic viral encephalitis, affecting all age groups with highest incidence of disease among children. The vast majority of JE infections are inapparent, only 1 in 25 to 1 in 1000 infections result in symptomatic illness. The variation could be due to number of factors including endemicity, exposure to mosquitoes, pre-existing antibodies to flaviviruses and virus strain differences (1). The virus is found in vast geographic area and a rising trend in JE cases and spread in new habitats and environment has been observed. It is endemic throughout Far East and South East Asia and recently, has spread to other non-Asian regions e.g. Papua New Guinea and the Torres Strait Islands of Australia (2). Approximately 3 billion people live in endemic regions and about 50,000 cases of JEV infection and 10,000 deaths are reported each year. Approximately 50% of survivors have permanent neuropsychiatric sequelae (3). Virus persistence in the human nervous system has been reported in 5% of patients with JEV associated encephalitis (4).

JEV is transmitted to humans by rice-field breeding mosquito, mainly Culex tritaeniorhynchus. Virus exists between mosquito and pigs or water birds. Man is a "dead-end" host and plays no role in perpetuating the virus. Variation of JE transmission patterns occur within individual countries and from year to year. In subtropical and tropical endemic areas, risk is present throughout the year with occurrence of sporadic cases, but is accentuated during rainy season and early dry season when mosquito populations are higher. In temperate regions of Asia and the northern tropical regions, JEV is transmitted seasonally.

Presence of JEV has been shown serologically in different parts of India since the mid fifties (5). The virus was first isolated in 1958 from three sporadic cases of encephalitis in Tamil Nadu (6). The first major epidemic occurred in 1973 in Bankura and other districts of West Bengal (7). Subsequently, number of JE epidemics have been reported from states of Karnataka, Uttar Pradesh, Bihar, Assam, Goa, Kerala and Haryana (8-10). At present, JE is not only endemic in many areas, it is also spreading to naïve non-endemic areas.

The Virus :

JEV is an enveloped; plus-sense single stranded RNA virus, approximately of 11 kilobase, and is antigenically related to other flaviviruses including dengue. It contains several structural (capsid - C, premembrane - prM and envelop-E) and non-structural (NS1 to NS5) polypeptides, which are encoded by a single, long open-reading frame (ORF). Nucleotide sequence analysis of prM and E gene has suggested that there are five JEV genotypes (11,12). Though JEV isolates are grouped in distinct clusters within each genotype, but no clear distinct geographical boundaries appear to exist between the distinct clusters (11). Complete genomic sequence of the JE strains GP78, isolated at Gorakhpur (8) in Northern India and P20778 isolated from

Vellore in the Southern India are phylogenitically closer to the Chinese SA14 isolate (13) than to the Nakayama strain from Japan.

Pathogenesis:

Following an infective mosquito bite, the viral replication may occur locally and in regional lymph nodes or vascular endothelial cells. The incubation period is about 5-15 days. After hematogenous spread in the host, JEV replicates in number of organs and generates a rapid inflammatory response including peripheral neutrophil leucocytosis and mononuclear and polymorphonuclear leucocytosis infiltration in extra neural tissue (14). Clinically, the infection of JEV results in increased levels of cytokines such as macrophage derived chemotactic factor (MDF), TNF-α and interleukin (1L-8) in the serum and cerebrospinal fluid (15-17). The low levels of inflammatory mediators appear to play a protective role (18) where as increasing concentrations of cytokines are related to severity of illness (16). Viral invasion of central nervous system (CNS) occurs probably via vascular endothelial cells (19). Certain neurotransmitter receptors are involved in the binding of JE virion to the cells in CNS. The response to cerebral infection with JEV in mice is characterized by significant recruitment and extravasation of immuno inflammatory cells, predominantly macrophages, T cells and neutrophils, to sites of viral replication in the brain (14,19). Immuno histochemistry of JE infected human brain tissue indicates the presence of viral antigen in the thalamus, hippocampus, substantia niagra and medulla (20). The host response to infection is central to the effective control and ultimate clearance of invading pathogens.

JEV infection ranges from a febrile headache syndrome to an acute and possibly fatal encephalitis. Neurological sequelae is present in about 30 – 50% of the survivors (21). Viral persistence in the human nervous system has been reported in approximately 5% of patients with JEV associated encephalitis (4,22).

Clinical Features:

JEV being a neurotropic virus, targets the CNS and the clinical picture varies according to the severity of CNS involvement age, nutritional status of the affected individual and on the degree of neuronal maturity as well as of the presence of intercurrent infections (23). In endemic areas children below 15 years of age are most commonly affected. The incubation period in man varies between 1 to 15 days. The course of JEV infection can be divided into 3 stages. The prodromal stage (1-5 days), acute encephalitic stage (6-10 days) and convalescent stage. The prodromal stage is marked by abrupt onset of fever, malaise, anorexia, headache, nausea and vomiting without involvement of the CNS. Although spontaneous recovery from this stage is known, the disease may progress to the acute encephalitic stage which is characterized by signs of involvement of CNS. Onset of this stage is rapid with fever, headache, nuchal rigidity, convulsions, altered consciousness progressing to coma. This is followed by appearance of focal meningeal and extrapyramidal signs, such as dull mask like face, muscular rigidity and cranial nerve palsies. During convalescent

stage neurological signs tend to improve and patient either becomes normal or may develop sequelae. Features suggesting of Parkinsonism (24) or Guillen-Barre Syndrome (25) may occur.

Immune Response:

The host defence in JEV infection is mediated by the cooperative activity of various components of phagocytic cells and different subsets of B and T effecter cells. Though the relative contribution of individual components has not been well understood, the innate immune response, after primary JEV infection, plays an important role in the restriction of infection or clearance of invading pathogens. JEV infection stimulates macrophage derived neutrophil chemotactic factor (MDF) production (15), which is involved in early host defence (18,26). Humoral immunity is an important component of immune response to JEV. First, there occurs IgM response usually within 7 days of infection. Early appearance of IgM antibodies in CSF has been correlated with favourable outcome in JE (27). This is followed by appearance of IgG antibodies. Experimental studies in mice have shown that passive transfer of anti JEV polyclonal as well as monoclonal antibodies provide protection against JEV infection (28,29), while presence of JEV specific immune complexes in CSF has been associated with fatal outcome (20).

The development of cell mediated immunity has been demonstrated experimentally in mice and linked with recovery from JEV infection as adoptive transfer of immune spleen cells provides protection against JEV challenge (28). Induction of JEV specific memory T cells after primary JEV infection has been demonstrated (30,31). JEV also generate protective cytotoxic T lymphocyte response (32)

Diagnosis:

In JE, during acute encephalitic stage peripheral blood counts show leucocytosis with neutrophilia. There is elevated cerebrospinal fluid (CSF) pressure. CSF examination shows marked pleocytosis (cell count 10 to 980 X 10^6/L), mild elevation in protein level and normal glucose concentrations. The electroencephalogram (EEG) shows diffuse theta and delta waves burst suppression and epileptiform activities, but these features are non specific. Brain computed tomography (CT) and magnetic resonance imaging (MRI) techniques may be helpful in distinguishing JE from herpes encephalitis (33).

Confirmatory diagnosis of JE can be carried out either by virus isolation or demonstration of antigen or by serology in CSF, blood or other specimens. Virus isolation from blood is rare, however virus could be isolated from CSF during acute phase of illness or from brain tissue in fatal cases by inoculation of specimens intracerebrally into infant mice, or various cell cultures or toxohynchites splenders larvae (34). Identification of virus is carried out by neutralization test or indirect immunofluoroscence test using JEV specific monoclonal antibiodies (35) or ELISA test. The isolation of virus is correlated with poor prognosis (27). A number of sensitive tests described for rapid antigen detection in CSF are reverse passive haemagglutination test for detection of soluble antigen (36) on immunofluoroscence tests for detection of cell bound antigen (35).

Conventional serologic tests are haemagglutination inhibition (HAI), neutralization and complement fixation tests. Demonstration of four fold or greater rise in HAI antibody titres in acute and convalescent serum samples is widely used antibody detection method for JE diagnosis. Cross reactive antibodies with other arboviruses make serodiagnosis difficult. IgM antibody capture ELISA (Mac-ELISA) is the method of choice to demonstrate virus specific antibody in CSF / blood in early phase of illness. Nitrocellulose membrane based IgM capture dot enzyme immuno-assay (Mac DOT) and antibody radioimmuno assay are some latest antibody detection methods. A reverse transcriptase polymerase chain reaction (RT-PCR) has been used for rapid detection and identification of JEV (37).

Treatment & Control:

For JE treatment no specific antiviral therapy is available. It is essentially symptomatic. Maintenance of fluid and electrolyte and good nursing care is essential. Control of pyrexia, seizures and cerebral edema is necessary. Control measures are aimed at the vector, vertebrate animal host or susceptible human population. Vector control can be achieved by reducing mosquito breeding which include draining out of stagnant water and spraying or fogging with insecticides such as pyrethrum and malathion.

Vaccines:

The main strategy for control of JE is vaccination of susceptible human population, which involves three main aspects such as age of the individual, cost of the vaccine and immune response to the JE. Three main types of vaccines are currently in use and different approaches for development of new effective vaccines are in progress.

Mouse brain inactivated vaccines:

The inactivated mouse brain derived vaccine is prepared by injection of the JE virus into infant mouse brain. The purified vaccine is in use since 1968. It is effective in practically eliminating the disease. As the two strains of JEV may be in circulation, a bivalent vaccine has been developed in 1984. Subcutaneous injection of vaccine of 1ml in adults and 0.5 ml in children below 3 years of age is recommended. For effective protection three doses are recommended over a period of 30 days (days 0, 7 and 30), followed by a booster one year later and subsequent vaccination every 3-4 years in endemic areas.

Cell culture derived inactivated vaccine:

An effective inactivated cell culture vaccine (Chinese SA-14-2 strain) for the control of JE has been used in China since 1967. Inactivated JE vaccine is produced in primary hamster kidney cells and is administered to children aged 6-12 months in two doses spaced 1 week apart followed by a booster dose in second year (38)

Live attenuated vaccine:

An effective, inexpensive live attenuated JE vaccine has been developed using SA 14-14-2 strain of JEV in primary hamster kidney cell line and is in use in China. It is safe with few untoward reactions. The effectiveness of two doses of vaccine given 1 year apart is 97.5% and that

of one dose is 80%. Protection lasts atleast for 5 years (39). Various studies are directed towards the development of the newer JE vaccines. Pox virus (Canary pox and vaccinia) based JE recombinant vaccine has been constructed (40). In recent years subunit vaccine have been developed to induce protective immune response against JE. Immunization with recombinant plasmid DNA vaccine containing the JEV prM and E genes elicit neutralizing antibodies against JE virus (41). A chimera vaccine (ChimeriVax-JE) in which the structural proteins prM and E of yellow fever (YFV 17D) are replaced with those of JEV SA 14-14-2 vaccine strain is under evaluation as a candidate vaccine against JEV (42). However, all these approaches are in the experimental stage and need further evaluation.

Virus - host interplay during Japanese encephalitis virus infection :

In one of our initial study of an epidemic of encephalitis which occurred in Eastern districts of UP during post monsoon season in 1978, involving large segment of population with high fatality, we investigated large number of the cases admitted in different hospitals of Gorakhpur and Deoria. The etiological diagnosis of JE in these patients was established either by isolation of virus from CSF or brain or by demonstration of antigen in CSF by indirect immunofluoroscence or by the presence of virus specific IgM in serum or four fold or greater rise in antibody titre in serum.

Leucopenia with lymphopenia is a frequent feature of most of the viral infections, while JEV infection, results polymorphonuclear leucocytosis with variable effect on different components of the peripheral blood leucocytes (44). To validate the clinical observations, mouse model was developed. One set of our studies had shown that after JEV entry the host virus replicates in a number of organs and generates a rapid inflammatory response with mononuclear and polymorphonuclear cells infiltration in various tissues (14). Figure-2 shows the total leucocyte counts and percentage neutrophils at different periods in the peripheral blood of JEV inoculated mice (i.p.) JEV infection induced leucocytosis with neutrophilia. The mean count in JE infected mice was 20,000+ 580/mm^3 on day 11 after inoculation, while in normal controls it was 8,400+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%) (43).

The inflammatory response within the central nervous system in viral encephalitis is regulated through a network of cytokines and chemokines. Chemokines are a family of small (≈8 to 14 KD), structurally related chemoattractant cytokines that are produced upon activation by different cellular sources like T cells , monocytes, microglia, astrocytes, fibroblasts, epidermal and endothelial cells (46). Chemokines are important regulators of leucocyte trafficking to sites of immune challenge or tissue damage (47,48). The chemokine fall into four categories which are defined by a cysteine motif: CXC, CC, XC and CX_3, where C is cysteine and X is any aminoacid residue. The CXC chemokine sub family includes interleukin-8.

The mechanism of neutrophil leucocytosis in Japanese encephalitis virus infection is not known. Present data demonstrate the production of a previously unrecognized neutrophil chemotactic cytokine secreted by the macrophages in spleen during JEV infection. Figure -3 shows that the maximum production of neutrophil chemotactic activity was obtained on day 6 following inoculation of 0.3 ml of 10 LD_{50} of JEV intraperitoneally (mean migration / hpf = 39+1.9). To delineate the cell type responsible for chemotactic activity in vitro, normal mouse splenic macrophages, T and B lymphocytes ($5x10^6$ cells/ml) were cultured and stimulated with 10^3 LD_{50} of JEV. The chemotactic activity was obtained by JEV stimulated macrophages only while T and B lymphocytes supernatants failed to attract neutrophils. On purification of macrophage derived supernatants (MDF) on Sephacryl S-200 column (Fig. 4) and further on Pep-S column, it migrated as a single band of 10 k Da on polyacrylamide gel (15). The MDF reacted specifically with anti-MDF antisera on Western blot. MDF was found heat resistant and show no change after 4h incubation with proteases. It results distinct leucocytosis with neutrophilia after purified MDF inoculation in mice (Table1).

Further our studies have demonstrated that infection of JEV results in increased level of cytokines such as macrophage derived chemotactic factor and interleukin-8 (IL-8) in serum and cerebrospinal fluid (CSF) of JE patients (17), JEV stimulates human peripheral blood monocytes (hMDF) which secrete a chemotactic cytokine named as hMDF which generates a rapid inflammatory response including neutrophil leucocytosis. Figure-5 shows the presence of virus induced hMDF by immunoblot assay in more than 80% of acute phase sera of JE confirmed patients. The observation revealed that mortality rate increased with increasing concentrations of 1L-8 in the serum and cerebrospinal fluid in JE patients(Figure 6) (17).

In another set of experiments the role of MDF in the pathogenesis of JE and its effect on the integrity of the blood – brain barrier was studied. Our finding demonstrated that JEV along with MDF could cause an alteration in the permeability of the blood brain barrier resulting in the leakage of plasma protein bound Evans blue dye and radiolabelled erythrocytes in brain. Figure 7 shows [^{51}Cr]-labeled erythrocytes and leakage of Evans blue dye in the brain of mice at different periods after JEV inoculation (i.c.). The maximum erythrocyte and dye protein leakage occurred at day 6 after JEV infection, while with MDF the leakage was at 1 hour post inoculation (figure 8), which directly correlated with the maximum production of MDF in vivo. Complete restoration of the integrity occurred by 4h (19). In other set of experiments, the effect of MDF in micro-vasculature during JE infection was studied. Figure 9 shows the intradermal inoculation of MDF in rabbits caused [^{51}Cr]-labeled neutrophil emigration and peak accumulation of neutrophils into the injected sites at 1 hour following MDF inoculation (49).

Further observations had revealed significant fall in serum iron levels during JEV infection in humans (35) and in mice (14). To address the mechanism for

hypoferraemia our studies revealed that JEV induced MDF was associated as possible regulator for the lowering of the serum iron levels in mice. The findings presented in table 2 showed that purified preparation of MDF caused significant depression (42%) in serum iron levels as measured after 24 and 48 hours after inoculation. Further, the iron staining of MDF inoculated mice spleen has revealed, increased iron deposition within splenic macrophages at 24 and 48 hours, which gradually declined at 72 hours after MDF injection (14)

Host defence mechanism during JE infection:

The host response to infection is central to the effective control and ultimate clearance of invading pathogen. The response to JEV infection in mice is characterized at the pathologic level by significant recruitment and extravasation of immuno inflammatory cells, predominantly macrophages, T cells and neutrophils to sites of viral replication in spleen as well as the brain (14,19). The neutrophil and macrophages are key effector cells involved in early host defence. The role of neutrophils in antiviral defence has been scarcely studied. It may act as effector cells in antibody - dependent cell cytotoxicity (ADCC) as seen in herpes simplex virus infection (50) or release of interferon like substances in response to certain viral antigen (51), or degrade the virion (52). One of our studies was undertaken to explore the contribution of neutrophils towards host defence against Japanese encephalitis virus. The ability of neutrophils to degrade the phagocytosed JE virion, via triggering the respiratory burst and generation of toxic radicals had been investigated.

In order to establish the interaction of neutrophils with particulate (virus) or soluble substances (MDF) in activation of oxidative and non-oxidative mechanisms, the phagocytic activity of normal neutrophils, exposed to purified JEV and MDF at different time periods was measured. Figure 10 shows that MDF stimulation increased the phagocytosis of virus at 60 minutes as compared to JEV alone. The phagocytosis triggers the activation of the oxidative signals with generation of superoxide anion. Neutrophils were stimulated in vitro with 5 μg of MDF and superoxide generation was evaluated. Neutrophils treated with MDF elicits rapid (maximum at 30 sec) respiratory burst (mean value = 0.23+ 0.08 nM/min/10^6 cells; $p<0.05$) as compared to control (mean value = 0.12 + .001 nM/min/10^6 cells). A series of experiments showed that subsequent formation of hydrogen peroxide via activation of NADPH with concomitant release of myeloperoxidase which peaked on day 7 post JEV infection coinciding with the maximum production of MDF. The respiratory burst was abrogated by staurosporine (protein kinase C inhibitor) indicating that neutrophil activation and signal transduction by MDF are dependent on protein kinase C (26,53). We have also observed that it acts in Ca-dependent manner. The data indicate that MDF showed significantly increased capacity for induction of respiratory burst and appears to be the central mediator for production of oxygen metabolites of neutrophils during JEV infection (54).

Degradation of viral protein and RNA

Further experiments were planned to explore whether the phagocytosed virus is actually degraded by neutrophils. In studying degradation of viral glycoprotein, neutrophils ($3x10^7$ cells / ml) were incubated with 30 µl of purified (^{35}S) Methionine labeled virus in presence of serum for 1 h, treated with TX - 100 and layered onto 6% (W/V) sucrose. The time dependent degradation of the viral protein in the neutrophils was measured and compared with MDF prestimulated and similarly treated neutrophils. The findings summarized in Figure 11 show that significant degradation of viral protein occurred at 120 min (44 +5%, $P<0.05$). The percent degradation at the same time was higher (62 + 3%) in cells prestimulated with MDF. However, a partial inhibition of viral protein degradation enhanced by MDF was observed following pre treatment of cells with staurosporine. To confirm viral protein degradation SDS-PAGE was performed which showed presence of low MW proteins as compared to normal JEV protein pattern. For studying the degradation of viral RNA the neutrophils ($3X10^7$ cells/ml) were incubated with (^3H) - Uridine labeled virion and TCA precipitable radioactivity was measured 60 and 120 min later. Findings summarized in Table 3 show substantial viral RNA damage at 60 minutes in neutrophils. The percentage viral degradation was significantly higher at 120 minutes of incubation ($P<0.05$) (26).

Antiviral effect of Nitric oxide

Nitric oxide (NO) has emerged as an important intra and intercellular regulatory molecule, which plays important roles in immunological pathways in the mediation of central and peripheral nervous system functions. NO has been implicated as a mediator of anti viral host defence. Many cells (macrophages, neutrophils, neurons etc.) are able to produce NO through the enzymatic conversion of L-arginine to L-citrulline by nitric oxide synthase (NOS), in presence of NADPH. Three isoforms of NOS have been identified. Two are constitutively expressed Ca^{2+} dependent forms (cNOS), found in a variety of cell types, including neurons (nNOS) and endothelial cells (ecNOS). The other isoform, an inducible Ca^{2+} independent form (iNOS) has potential to generate NO for extended periods of time. iNOS expression is significantly induced by lipopolysacchride or cytokines, TNF-α and IFN-γ, in a variety of immuno inflammatory cells, including macrophages (55). iNOS may play a role in the antimicrobial and antitumor functions of the immune system. Cytokine inducible NOS is expressed by microglia and astrocytes, which implies a possible role for the enzyme in central nervous system host responses. A series of experiments were performed to study the ability of JEV and JEV induced MDF to modulate NO activity in brain and the possible antiviral effect of NO during JEV infection. Figure 12 illustrates that splenic macrophages of JEV infected mice produce maximum NO in vivo at day 7 post infection. MDF induced NO production was dose dependent and maximum at 60 minutes after MDF treatment. The response was sensitive to anti MDF antibody treatment and the nitric oxide synthase inhibitor N^G - monomethyl -L-arginine (L-NMMA). In order to ascertain the antiviral

role of NO on JEV infection, mice were pretreated with L-NMMA followed by JEV inoculation. Data summarized in Figure 13 show that L-NMMA treatment significantly increased the mortality in JEV infected mice as compared to control (56).

Nitric oxide synthase in JEV infection

The ability of JEV and JEV induced MDF to modulate nitric oxide synthase (NOS) activity in brain and tumor necrosis factor (TNF-α) and the possible anti viral role of NOS during JEV infection was investigated. The data presented in Figure 14 show that the total NOS activity in brain increased gradually from day 3 and reached a peak on day 6 (176.2+12.2 pmol/min/mg protein) in JEV infected mice as compared to controls ($p<0.001$). There was no significant alteration in the cNOS activity (99.3+ 8.9 p mol/min/mg protein) throughout the study period. The control mice, exhibited total NOS activity of 118.1+ 10.1 p mol/min/mg protein, cNOS activity of 5.2 + 0.6 p mol/min/mg protein. The regulatory role of MDF in the modulation of NOS activity in brain was evaluated and finding revealed that maximum NOS activity in brain was at 60 min p.i. and this increase was mainly due to the induction of iNOS. Figure 15 shows NOS protein expression by immunological analysis at different time intervals. A time dependent increase in NOS protein expression after MDF treatment was observed at 30 min, with maximum expression at 60 min post inoculation. No significant alteration in cNOS protein expression was observed. Pretreatment of JEV infected mice with L-NMMA increased the mortality, as evident from reduced mean survival time. Cytokines are potent modulators of iNOS during viral infections and play a pivotal role in regulating the protective immune response. The enhanced level of TNF-α observed in the early phase of JEV infection which correlated well with the enhanced activity of iNOS (18).

Antiviral effect of DDTC

Diethyldithiocarbomate (DDTC), a low molecular weight dithiol, has been described as an immuno modulator and shown to be effective in several disease conditions. Therefore, we studied the therapeutic aspect of DDTC in providing inhibition of JEV infection. DDTC tested at various doses (10-100 μmol /kg/; i.p.) revealed that administration at low concentration (10 μmol/ kg/; i.p.) prolonged the average survival time (AST) of mice infected with lethal dose of JEV. The low dose also provided >80% survival in sub-clinical (10^5 LD_{50}, i.c.) JEV infection (Figure 16). Administration of DDTC to JEV infected mice enhanced the inducible nitric oxide synthase (iNOS) activity in brain. Thus these studies demonstrate that early non specific protection against JEV is mediated by the co-operative activity of reactive oxygen and nitrogen metabolites (57).

Host defence against viral infection is a complex phenomenon. The antibodies and the cell mediated immune response produced after JEV infection (Figure 17, 18). A series of our studies have shown that in JEV infection the antibody and cell mediated immunity afford specific protection. It triggers transient protective cell mediated immune response and induces delayed type hypersensitivity (28).

The CD4 and CD8 cells are thought to be important. JEV has the ability to establish latent infection in mice (58), which is associated with defect in cell mediated immune response (59). JE virus also stimulates the formation of IgM and IgG antibodies both in human and experimental animals with neutralization and haemagglutinating activity (Mathur et al, 1983a). Passive transfer of anti-JEV polyclonal or monoclonal antibodies provides protection against JEV infection (28,29). The accelerated generation of secondary immune response following JEV challenge in latently infected mice due to the presence of antigen specific memory B and T cells is seen in JEV infection.

Transplacental transmission of virus during JE infection

Few viruses have the ability to establish simultaneous infection in both the pregnant female and the foetus in utero. During an extensive epidemic of JE in Gorakhpur in 1978, the human transplacental transmission of virus has been demonstrated for the first time (60). Recently it has been described for West Nile virus as well (61), therefore ultrasonographic examination of the foetus is now recommended if maternal illness due to West Nile virus occurs during pregnancy (62). We have developed the JEV mouse model to validate the clinical observations. The experimental studies in mice have shown the variable effects of JEV virus infection on the foetus at different periods of gestations. JEV infection during first week of gestation led a significantly higher number of abortions, still births and neonatal deaths, as compared to infection during the third week of gestations (63). Development of hydrocephalous, runting or retarded growth in baby mice has been noted three to four months after birth. JEV can establish persistent and latent infection in humans as well as in mice (58, 22) and could transmit the virus to the foetus in consecutive pregnancies (63). Latently infected mice showed poor cell mediated response (59). Persistence of JEV in cerebrospinal fluid upto 110 days post infection can occur along with high titres of IgM and neutralizing antibodies (20).

Conclusion and future directions

The emerging picture based on our research findings exhibit complex interactions between functional characteristics of virus and host immune system mediated in part by Macrophage – derived factor (MDF). These interactions between virus and host have been the subject of many classical studies that have now progressed to the molecular level as well. However, this model should also be viewed cautiously in light of regional differences in the genetic makeup of the human population and the circulating viral strains. There are many secrets to these interactions that must be discovered. It is hoped that further research in the context of ongoing prospective studies, using newer molecular and immunological techniques will achieve this potential. As a better understanding of the immune response to JEV in the context of disease as well as vaccine-induced protection becomes available, the ability o control the growing worldwide burden of JE will likely be improved.

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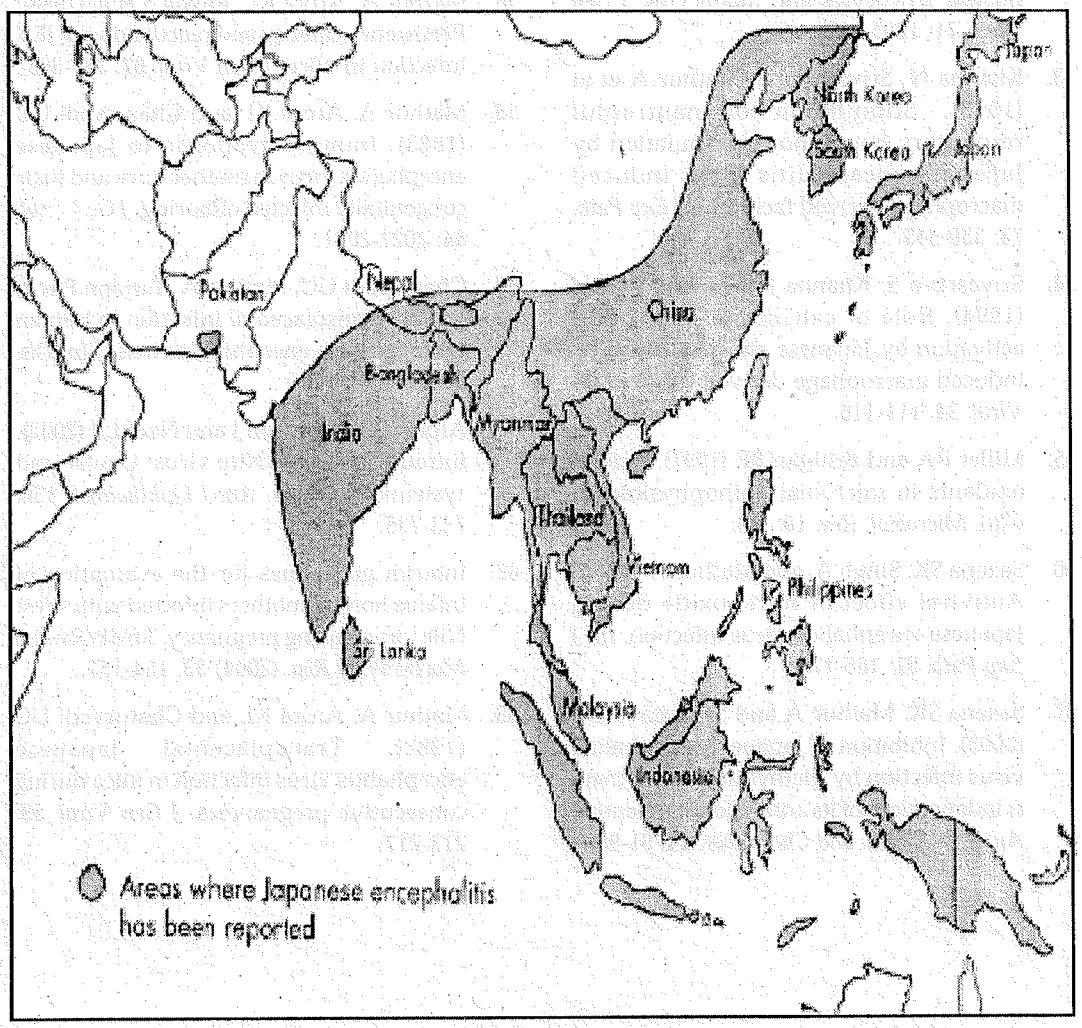


FIGURE 1. Showing areas where JE has been reported
(Courtesy : Tiroumourougane et al, 2002)

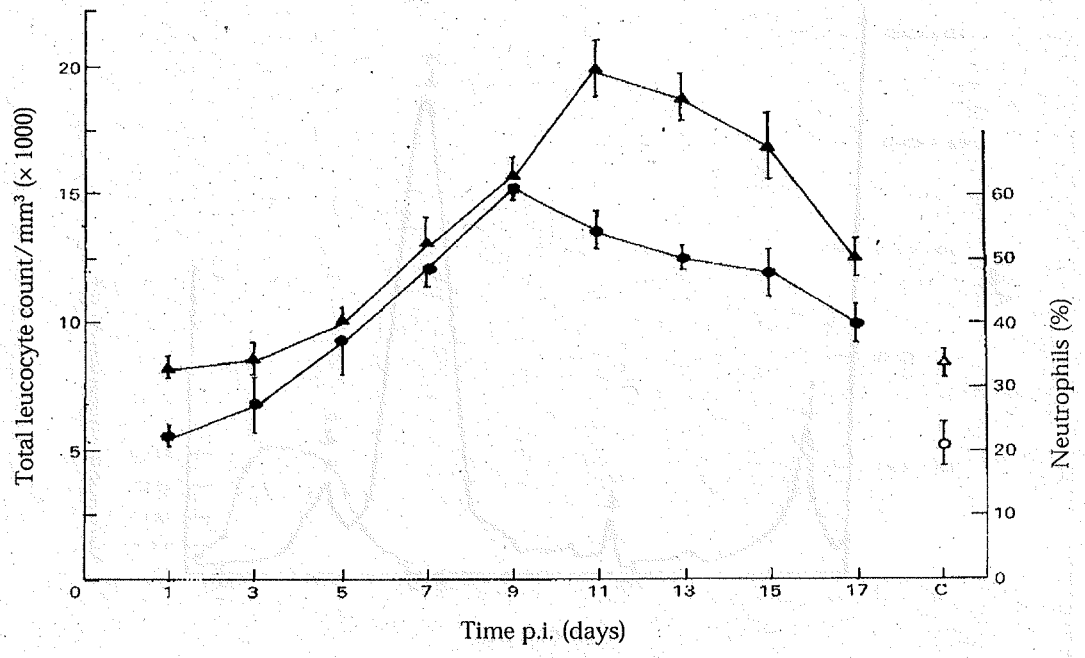


FIGURE 2. ▲ Total leucocyte counts and ● percentage neutrophils at different periods in the peripheral blood of JEV i.p. inoculated mice. Each value represents means ± s.e. from five mice.

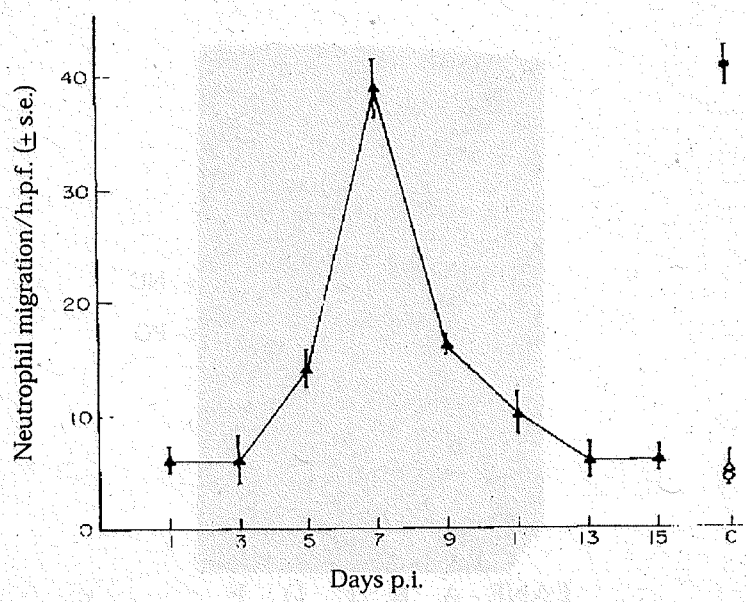


FIGURE 3. Spleen cell neutrophil chemotactic activity at different periods of JEV-primed mice (▲), normal mice (Δ). FMLP (10^{-7}) (●) was used as positive control and MEM (o) as negative control. Each sample was tested in triplicate with neutrophil migration counted in five to seven high power field.

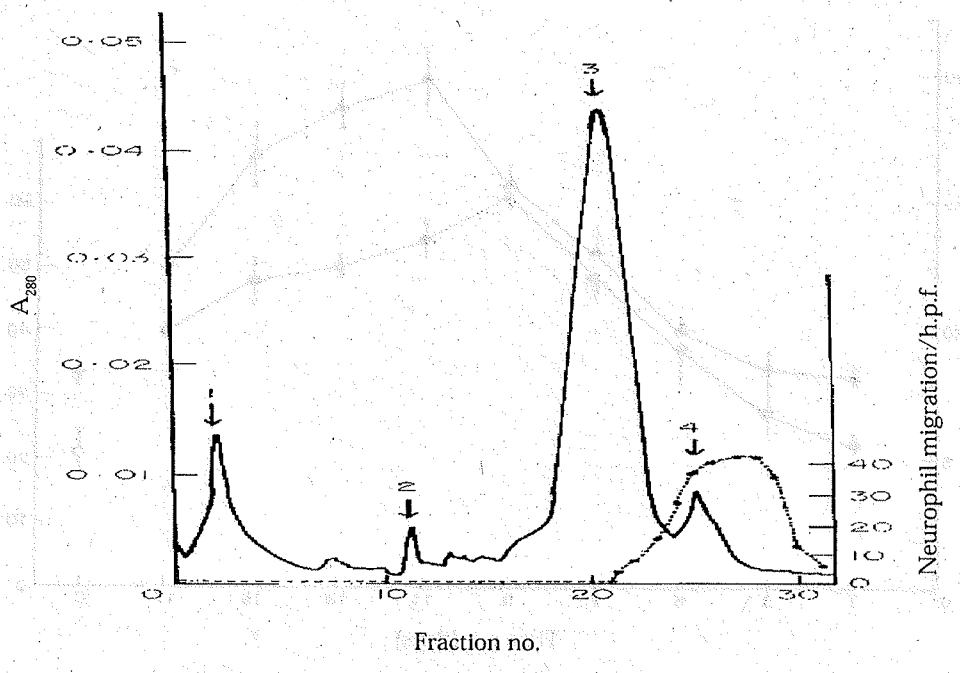


FIGURE 4. ▲ Purification of macrophage-derived factor by low pressure liquid chromatography (—) and neutrophil chemotactic activity (- - -)

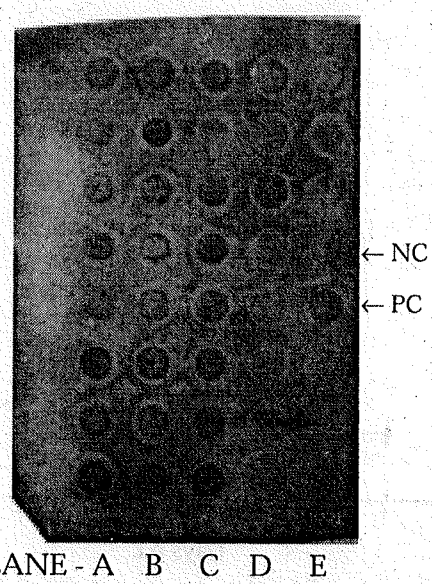


FIGURE 5: Immuno dot blot assay of serum and PBMC culture supernatants of JE confirmed cases.

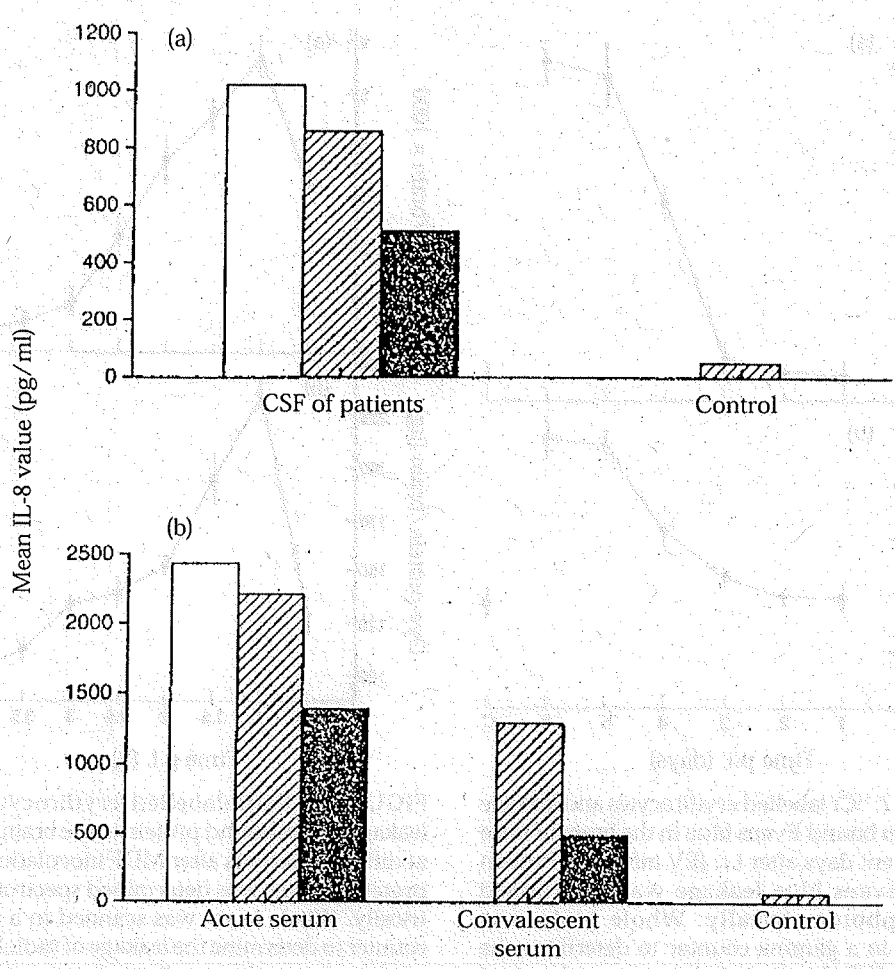


FIGURE 6: IL-8 levels in (a) CSF and (b) serum from patients with JEV infection : fatal cases (□), patients with prolonged illness (▨), and patients who recovered completely (■). Controls consisted of CSF (▨) from patients with symptoms other than acute encephalitis and serum (▨) from normal healthy individuals. Data are expressed as mean and SD.

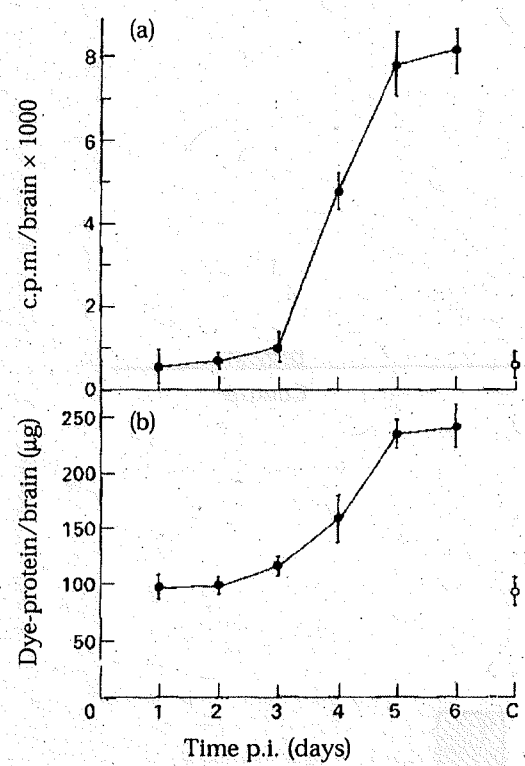


FIGURE 7: ^{51}Cr labelled erythrocytes and leakage of protein bound Evans blue in the brain of mice on different days after i.c. JEV infection. Protein bound Evans blue leakage was determined spectrophotometrically. Whole brain was scanned in a gamma counter to determine the leakage of ^{51}Cr labelled erythrocytes, and c.p.m./brain is presented. Each value represents mean ± s.e. from 12 mice.

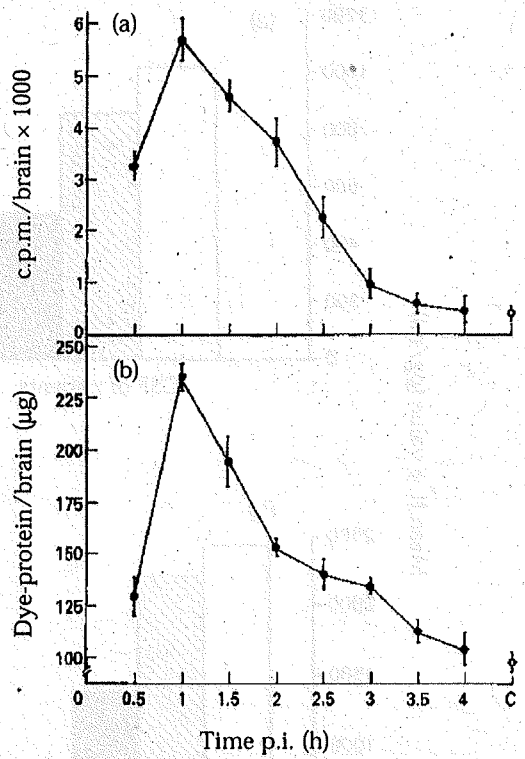


FIGURE 8: Radiolabelled erythrocytes and leakage of dye bound protein in the brain of mice at different periods after MDF inoculation. Dye-protein leakage was determined spectrophometrically. Whole brain was scanned in a gamma counter to determine the leakage of radiolabelled erythrocytes, and c.p.m./brain is presented. Each point represent mean ± s.e. from 12 mice.

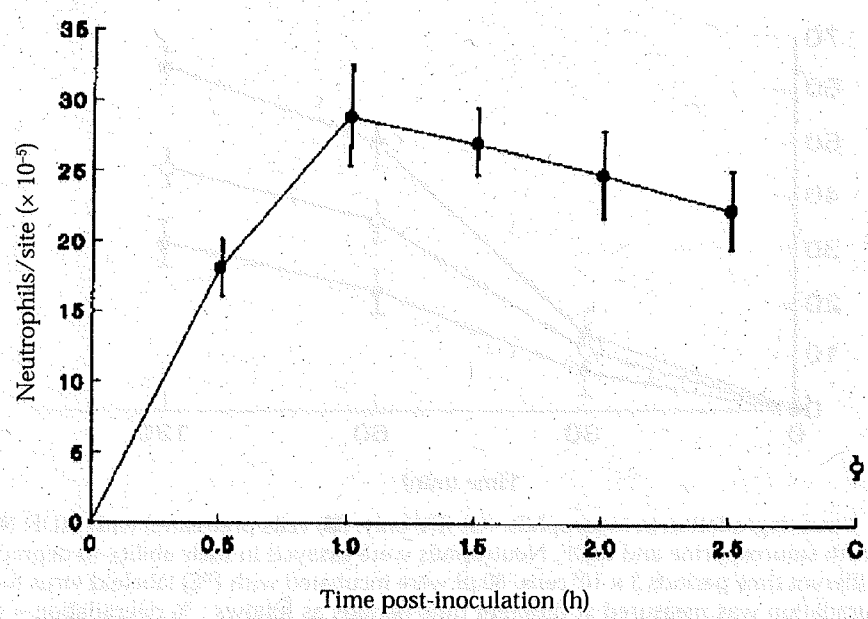


FIGURE 9: [^{51}Cr]-labeleld neutrophil accumulation at different periods after intradermal injection of MDF in rabbit

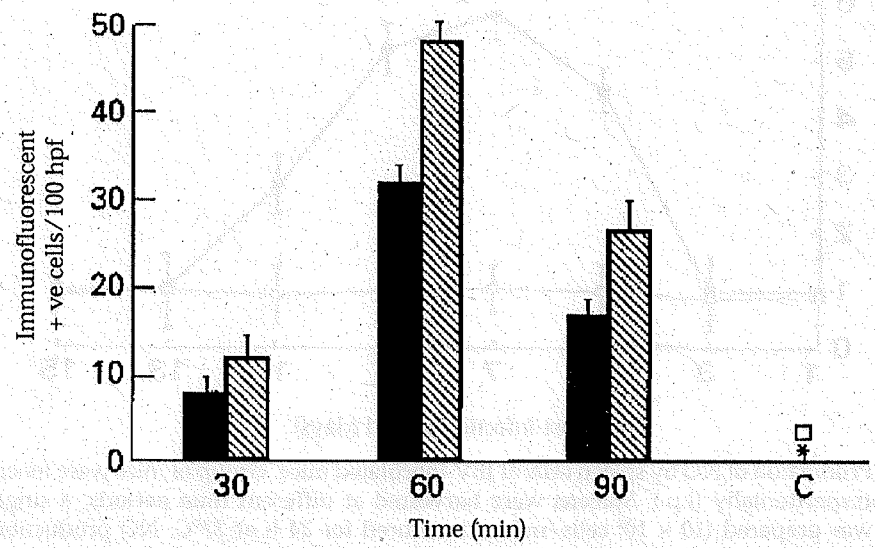


FIGURE 10: Demonstration of number of JEV positive immunofluorescent cells at different time periods after JEV (■) or costimulation with MDF and JEV (▨). Control cells (C) were stimulated either with MDF (□) or normal macrophage culture supernatant (*). IF positive cells were counted in 100 high power fields (hpf). Values are presented as mean ± SE of triplicate experiments.

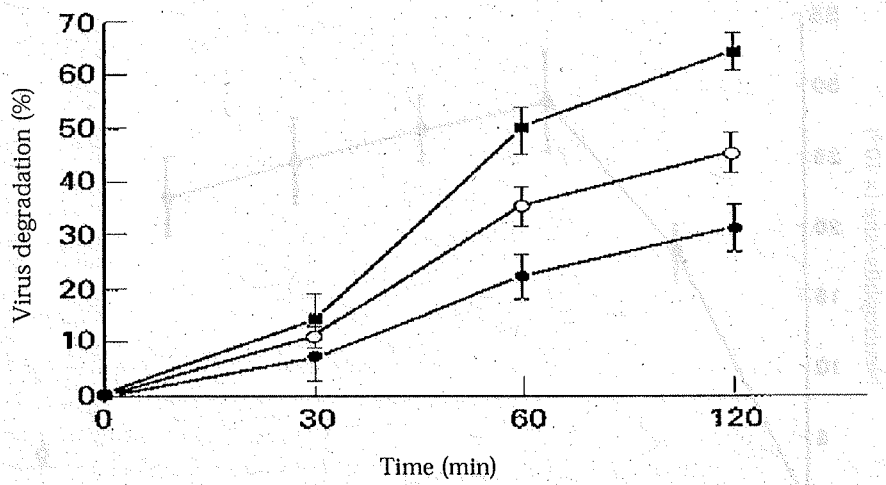


FIGURE 11: Virus degradation in neutrophils. (O) JEV only; (■) cells pretreated with MDF; (●) cells pretreated with staurosporine and MDF. Neutrophils were assayed in their ability to degrade viral protein at different time periods 3×10^7 cells/60 µl were incubated with (^{35}S) labeleld virus for 1 h at 37°C. % degradation was measured at different time periods as follows : % degradation = cpm in supernatant × 100/cpm in pellet + supernatant. Each value represents mean ± SE of three experiments.

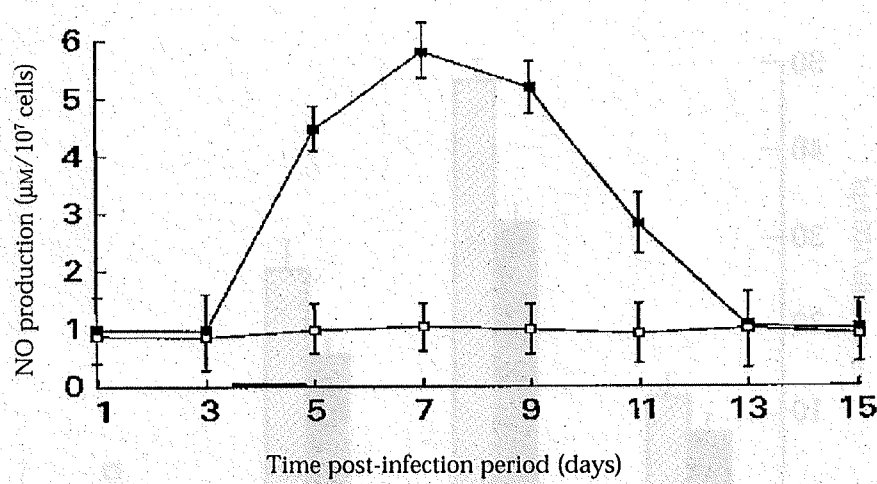


FIGURE 12: Production of NO by spleen cells of JEV-inoculated mice. Group of mice were inoculated with JEV intraperitonially (i.p.). Spleens were harvested at different time periods, a single cell suspension was prepared (10×10^6 cells/ml) and cultured for 24 h at 37°C. NO production was assayed in the cell free culture supernatants (■) as described in Materials and methods. Control (□) mice were inoculated with normal mouse brain suspension. Values are presented as A.M. ± SD from 10 cultures.

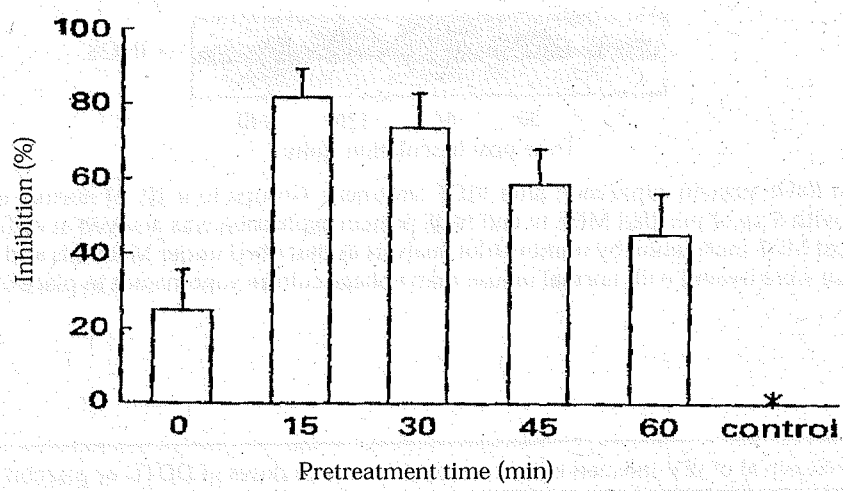


FIGURE 13: Inhibition of NO production by treatment with N^{G} monomethyl-L-arginine (L-NMMA). Normal mouse macrophage cultures were pretreated with 100 μM L-NMAA for indicated time periods followed by inoculation of 5 μg MDF for 60 min at 37°C. Control group of cells (*) were treated with MDF only. Cells treated with L-NMMA for different time periods were used for background values. NO production was assayed as described in Materials and methods. Results are presented after deduction of background values as A.M. ± SD for eight cultures.

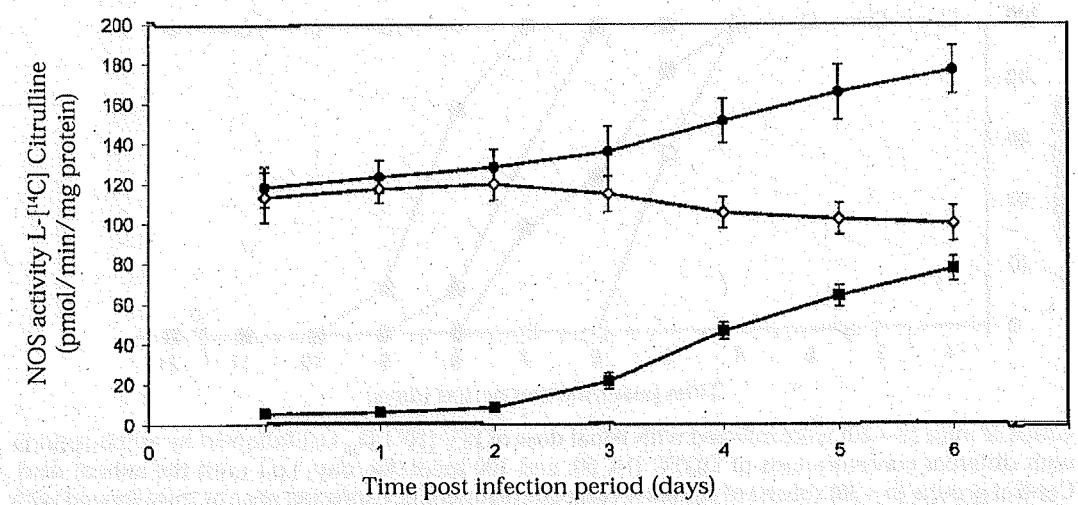


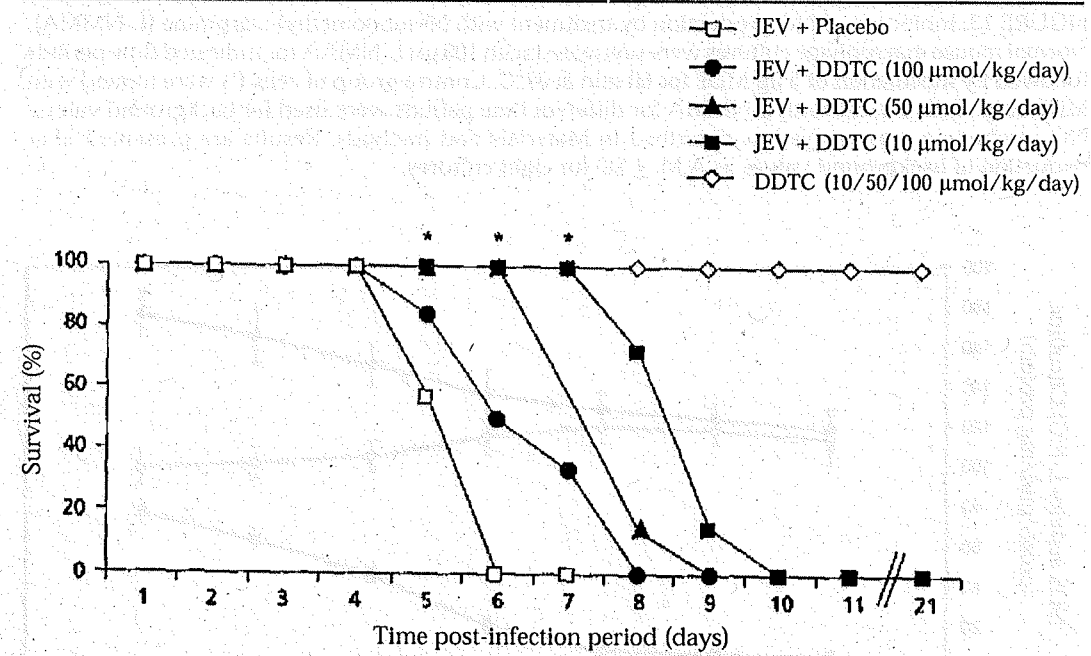
FIGURE 14: JEV-induced NOS activity in brain. Groups of mice (n = 10) were inoculated with 0.025 ml of 10^2 LD_{50} JEV (ic) or normal mouse brain suspension (for control). NOS activity was assayed in brain on different days p.i. as described under Materials and Methods. The NOS activity was expressed as A.M. ± SD of five experiments. (●) Total NOS. (◇) cNOS, (■) iNOS.

C 30 60 120 240

Time post inoculation (min)

FIGURE 15: iNOS protein expression after MDF treatment. Groups (n = 10) of normal mice were inoculated with 5 µg of purified MDF iv and NOS protein expression was assayed at different time intervals post MDF inoculation by immunoblot analysis as described under Materials and Methods. Control mice were treated with normal mouse macrophage culture supernatant in place of MDF.

FIGURE 16: Survival of JEV-infected mice treated with various doses of DDTC or placebo



Grops of mice (n = 20) were infected with lethal dose of JEV (10^2 LD_{50} i.c.) followed by adminisration with different concentrations of DDTC (10, 50, and 100 µmol/kg/day, i.p.) until the animal died. Control groups (n = 20) consist of similarly placebo-treatment JEV-infected mice or mice treated with different concentrations of DDTC alone. The survival rate of mice was monitored daily for 3 weeks.

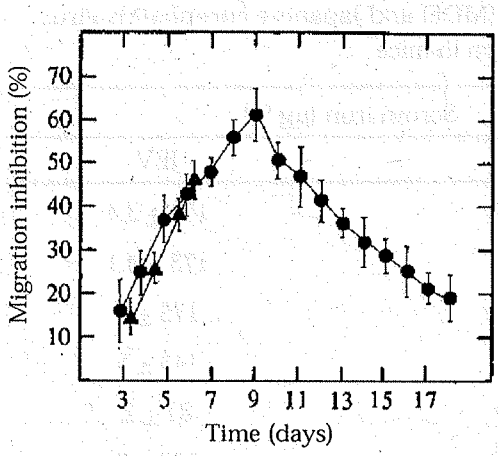


Fig. 1

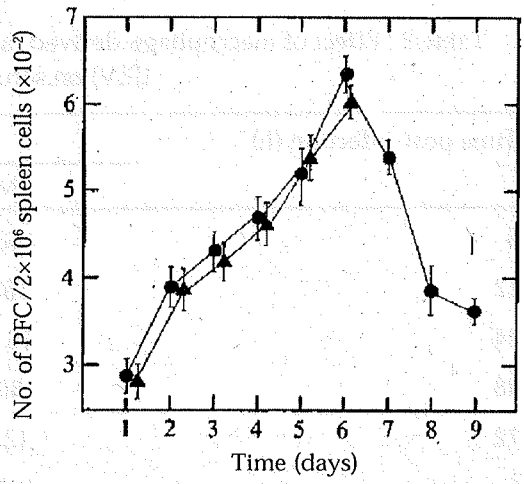


Fig. 2

FIGURE 17: Leukocyte migration inhibition of spleen cells of mice on different days after intraperitoneal (●) or intracerebral (▲) inoculation of JEV. Each observation represents the mean value with its standard error from multiple readings from five mice.

FIGURE 18: Antibody plaque-forming cells in the spleen of mice on different days after intraperitoneal (●) or intracerebral (▲) inoculation of JEV. Each observation represents the mean value with its standard error from multiple slides from five mice.

Table 1 : Peripheral blood leucocyte counts in MDF inoculated mice

Time post inoculation (h)	Total leucocyte counts (per min^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 ± s.e.

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

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

Values are expressed as mean of five to seven serum sample ± s.e.m.

Table 3 : Degradation of viral RNA by neutrohils at different time intervals

Phagocytosis (min)	% Degradation	
	Test	Control
0	3 ± 0.9	2.9 ± 0.3
60	28 ± 9.1	3.1 ± 0.6
120	69 ± 17.0	3.3 ± 0.5

Degradation of viral RNA which was phagocytosed by neutrophils for different time periods. Results shown are TCA-soluble cpm as a percentage of the total associated cpm ± SE of triplicate experiments. The control values represent degradation of virus incubated in MEM alone at respective time periods. 0.069 ± 0.027