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Japanese encephalitis virus latency in peripheral blood lymphocytes and recurrence of infection in children

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SUMMARY

In a study group of 40 children who had been admitted to hospital with acute encephalitis, the disease was due to infection with Japanese encephalitis virus (JEV). Three children developed recurrence of disease 8–9 months later. No virus had been isolated from these three patients during the acute stage of their illness, but virus was recovered from all during the recurrence phase by co-cultivation of their peripheral blood mononuclear cells in primary mouse embryo fibroblast cultures. Virus was also recovered by co-cultivation of peripheral blood mononuclear cells collected 8 months after their acute disease from three out of eight randomly selected asymptomatic children within the study group but not from similar cultures set up from JEV-seronegative children used as controls. Virus was also isolated by co-cultivation of T lymphocytes of asymptomatic children as detected by indirect immunofluorescence or by inoculation in mice.

Keywords Japanese encephalitis virus T lymphocytes latent infection recurrence

INTRODUCTION

Japanese encephalitis (JE) is one of the major causes of acute encephalitis with significant mortality throughout South-East Asia (Umenai *et al.*, 1985). The outcome of JE virus (JEV) infection varies depending on the interaction of virus and the host. Clinical encephalitis develops in only one out of the 600–800 persons infected with JEV (Halstead, 1981). Experimental studies carried out in mice showed JEV replication in several organs after primary infection (Mathur *et al.*, 1986a). The target cells are macrophages and T lymphocytes (Mathur *et al.*, 1989). Primary infection in mice is followed by establishment of latent infection of T cells (Mathur, Kulshreshtha & Chaturvedi, 1989). Since no information is available on latent infection in humans it was thought important to investigate this phenomenon.

In mice JEV can be activated following immunosuppression despite the presence of antibodies *in vivo* (Mathur *et al.*, 1986a) or after co-cultivation of latently infected cells *in vitro* (Mathur *et al.*, 1989). We have recently noticed recurrence of JEV-induced disease in children. The persistence of JEV-specific IgM (Edelman *et al.*, 1976) and sequelae after JEV infection in humans have been well established (Schneider *et al.*, 1974). We defined latency in JEV as the condition in which no viral antigen is detected in peripheral blood mononuclear cells (PBMC) by immunofluorescence or by direct isolation techniques, but in which the latently infected cells express virus after co-cultivation. The recurrent disease was defined as the reappearance of symptoms in a previously seropositive patient along with increase in titre of antibody or presence of specific IgM antibody or virus isolation. Here we describe the latent and recurrent disease as a consequence of activation of latent JEV in children.

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MATERIALS AND METHODS

Lymphocyte separation

PBMC were isolated by Ficoll–Isopaque density gradient (Nyegaard, Oslo, Norway) centrifugation. Briefly, the macrophages were obtained from PBMC by incubating 2×10^7 cells for 2 h at 37°C in glass Petri dishes with MEM–HEPES containing 10% fetal calf serum (FCS) and antibiotics in the presence of CO_2 and collecting the glass-adherent cells. More than 90% of these cells were phagocytic and were considered as the monocyte/macrophage population. The non-adherent T and B lymphocyte populations were purified by filtration through a nylon–wool column by the technique of Julius, Simpson & Herzenberg (1973) as described earlier (Mathur *et al.*, 1988). The purity was checked by treating these cells with Leu 1 *pan*-T monoclonal antibody (Becton Dickinson, Heidelberg, Germany) or anti human IgG antisera as described previously (Mathur *et al.*, 1989). The purity of T lymphocyte preparation was $89 \pm 2\%$ and B lymphocyte purity was $97 \pm 1\%$.

Co-cultivation

Mouse embryo fibroblast cultures were prepared from 9–11-day-old pregnant mice negative for JEV antibodies. Peripheral

blood mononuclear cells, or T and B lymphocyte, or monocyte/macrophage-enriched subpopulations were co-cultivated on mouse embryo fibroblast (MEF). Cells (2×10^6/ml) in MEM-HEPES with 10% FCS were added to MEF (2×10^5 cells) and incubated at 37°C. Several cultures were set up for each cell subpopulation. The cultures were coded and examined on alternate days for virus isolation by intracerebral inoculation in mice or for JEV-specific antigen demonstration by indirect immunofluorescence as described earlier (Mathur *et al.*, 1990). Throughout the study for indirect immunofluorescence, the cultures were spotted in triplicate on teflon-coated slides and were screened for virus antigen carefully. The anti-JEV monoclonal antibody designated 98.9.5i was kindly provided by Dr E. A. Gould, Institute of Virology, Oxford, England, and was used at a dilution of 1/100 of mouse ascitic fluid in PBS. It reacted exclusively with JEV and did not react with any other flavivirus. The smears were screened under a Dialux 20 Leitz fluorescent microscope. The monoclonal antibody had been checked for absence of non-specific reaction with normal mouse embryo fibroblast cells.

Neutralization test

Neutralization tests were carried out in infant mice by titrating the virus against immune sera of JE, West Nile (WN) and dengue type 2 viruses as described previously (Mathur *et al.*, 1982). The immune sera were provided by the Director, National Institute of Virology, Pune, India.

Serological study

The haemagglutination inhibition (HAI) antibodies were estimated by the technique of Clarke & Casals (1958) using a microtitre method as described earlier (Mathur, Arora & Chaturvedi, 1983). Freeze-dried JEV antigen was kindly provided by the Director, National Institute of Virology, Pune.

Assay of IgM

The IgM antibodies in blood were measured by an antibody capture ELISA (Burke, Nisalak & Ussery, 1985). Hyperimmune anti-JEV globulin conjugated to peroxidase was kindly provided by Dr B. L. Innis, Armed Forces Research Institute of Medical Sciences, Thailand. The substrate solution contained OPD (Sigma, St Louis, MO). Optical densities (OD) were measured at 492 nm on a Titertek Multiskan reader. The sera were tested in duplicate at a dilution of 1/10. The test serum OD/negative control serum OD ratio (P/N) was calculated. Value of $P/N \geqslant 2$ was considered positive.

Study group

Two-hundred and fifty-eight consecutive patients aged 6 months to 12 years admitted with acute encephalopathic illness (acute, non-transient alteration of conciousness with or without fever, or other neurological symptoms) from August 1987 to December 1988 at G.M. & Associated Hospitals, Lucknow, India, were studied. The diagnosis of JEV infection was confirmed in 141 patients either by isolation and identification of virus from CSF, or by detection of viral antigen in CSF cells by indirect immunofluorescence using JEV monoclonal antibodies or by the presence of virus-specific IgM in serum or four-fold or greater rise in HAI antibody titre in serum. The case/fatality ratio in JEV confirmed cases was 36%. Sequelae following JEV infection were noticed in 27% of children (R.K., personal observation). Only those children were included in the study who recovered and were seropositive for JEV. The study group consisted of 40 asymptomatic children (aged 3–6 years) whose parents agreed to participate in this follow-up study. Five JEV non-immune children served as control.

The children were examined during acute stage (first 5 days after onset of illness), during convalescence (between 5 and 15 days) and during latent stage (6 months later, when they were free of virus or virus-specific IgM, and showed no rise in antibody titres). The children were asked to return as soon as any symptoms appeared. Three of the children developed mild confusion to alteration of intellect, convulsion and loss of memory within 8–9 months after infection. The severity of acute illness in these subjects was not significantly different from that of patients who recovered without recurrent symptoms. Eight months after JEV illness, eight asymptomatic children were randomly selected for the study. A proforma-directed history was taken and physical and neurological examination was performed on hospital admission and at every visit.

RESULTS

Follow-up study of asymptomatic children

Forty children with confirmed diagnosis of JEV infection, who recovered completely, were included in this follow-up study. Six months later none of these children had detectable virus, whether PBMC were examined by indirect immunofluorescence or inoculated into mice. The serum had no IgM and showed no rise in antibody titres. Approximately 8 months after the primary JEV infection, eight children were screened for the presence of latent JEV. Table 1 shows the data of virus isolation, antigen demonstration in CSF and antibody titres in blood during acute and convalescent phase of infection in these children (subjects 1–8). These children were screened for the presence of latent JEV 8 months later by co-cultivation of PBMC with MEF (Table 2).

Recovery of JEV from PBMC of asymptomatic children

Peripheral blood mononuclear cells obtained from eight asymptomatic children and five seronegative controls were co-cultivated on MEF cultures. The activated virus was detected by indirect immunofluorescence using JEV-specific monoclonal antibody or by virus isolation in mice. The results of immunofluorescence were interpreted with great caution. In the PBMC cultures of three out of eight asymptomatic children the JEV antigen was detected. The infected cells showed a bright cytoplasmic fluorescence and were clearly distinguished from the uninfected or normal controls. A lag period of 10 days or more (maximum 18 days) was observed before virus activation, approximately 25–40% of the cultures from each of the children had latent virus. No virus was detected in PBMC of seronegative controls (Table 2). The activation of JEV in PBMC of the three children (subjects no. 1, 4, 8) who had positive immunofluorescent cells was confirmed after isolation of virus in mice. The neutralization tests were performed on all three isolates against JEV, WN and Dengue antisera.

All strains were maximally neutralized by JEV immune sera. Some cross-reactivity was also observed with WN and Dengue sera (Table 3). A good correlation was observed in the results of virus demonstration by immunofluorescence or by mice inoculation.

Table 1. Results of the diagnostic tests carried out at the acute phase of the disease on children who were asymptomatic or developed recurrence of Japanese encephalitis virus (JEV) infection

Subject no.	Age (years)	Sex	Acute CSF		JEV-Specific IgM†		Serum HAI antibody	
			Virus isolation	IF*	CSF	Serum†	Acute	Con.
Asymptomatic cases								
1	4·5	M	−	−	+	+	20	80
2	3	F	−	−	+	+	40	160
3	5	M	−	+	+	+	40	160
4	5	F	−	−	+	+	40	160
5	3·5	M	−	−	+	+	40	160
6	4	F	−	−	+	+	20	80
7	3	M	−	−	+	+	20	80
8	4	M	−	−	+	+	20	80
Recurrence of disease								
9	6	M	−	−	+	+	40	160
10	5	F	−	+	+	+	20	80
11	4	M	−	−	+	+	40	160

* Virus identified as JEV in CSF cells by indirect immunofluorescence (IF) using monoclonal antibodies. † IgM − P/N ratio ⩾ 2·0 in ELISA.

Table 2. Recovery of JEV from peripheral blood mononuclear cells of children 8 or 9 months after the initial illness

Subject no.	Earliest day of recovery after reactivation	JEV recovery No. positive/no. tested	
		IF	Mice
Asymptomatic			
1	12	2/9	2/10
2	0	0/10	0/10
3	0	0/10	0/10
4	11	4/9	3/10
5	0	0/9	0/9
6	0	0/10	0/9
7	0	0/10	0/10
8	12	3/9	4/10
Recurrent disease			
1	3	5/15	3/10
2	4	4/15	3/10
3	3	4/15	5/10
Controls ($n=5$)	0	0/40	0/35

Co-cultivation of PBMC subpopulations with MEF

In order to delineate the cell subpopulation of PBMC responsible for harbouring JEV, the enriched T and B lymphocyte and macrophage subpopulation of latently infected asymptomatic children were co-cultivated with MEF cells and screened on alternate days for the virus activation by indirect immunofluorescence. The results summarized in Table 4 show the presence of activated JEV in T lymphocyte of latently infected children between days 10–16 after co-cultivation. No virus was detected

Table 3. Results of neutralization test

Reference immune sera	Log neutralization index					
	Asymptomatic children (subject no.)			Recurrent disease (subject no.)		
	1	4	8	9	10	11
Dengue-2	0·5	0·7	0·6	0·9	0·6	0·4
JEV	2·4	2·8	2·2	2·6	2·2	2·8
WN	0·9	0·8	0·9	1·1	1·0	1·2

Table 4. Recovery of JEV from enriched subpopulation of PBMC of latently infected asymptomatic children

PBMC Subpopulations*	Earliest day of recovery after reactivation	JEV Recovered/no. tested	
		Asymptomatic	Control
T lymphocytes	12	10/31	0/17
B lymphocytes	0	0/31	0/17
Macrophages	0	0/31	0/17

* Peripheral blood mononuclear cells enriched subpopulation was obtained from three latently infected asymptomatic children who had positive cultures (subjects no. 1, 4 and 8).

Table 5. Serum antibody response in children at various times after JEV infection

Subject no.	Antibody 6 months post-infection: Serum JEV IgM	Antibody 6 months post-infection: HAI titres	Antibody before virus activation or after recurrence of infection: Serum JEV IgM	Antibody before virus activation or after recurrence of infection: HAI titres
Asymptomatic				
1	0	80	0	80
2	0	80	0	80
3	0	40	0	20
4	0	80	0	80
5	0	80	0	80
6	0	40	0	20
7	0	40	0	40
8	0	40	0	40
Recurrent disease				
9	0	80	0	80A 320C
10	0	40	+	40A 80C
11	0	40	+	40A 160C
Controls ($n=5$)	0	10±0*	0	10±0*

A, acute serum; C, convalescent serum; serum JEV IgM – (0) – P/N ratio < 2 in MAC ELISA.

* All cases.

in B lymphocytes or macrophages of these children or from any enriched subpopulation of seronegative controls.

Study of children with recurrence of disease

Three of the 40 children with confirmed diagnosis of JEV (subjects 9–11 in Table 1) were included in the follow-up study, they developed recurrent symptoms after 8–9 months of recovery. These patients complained of mild confusion convulsions and loss of memory. No virus was isolated from sera of these patients by intracerebral inoculation in mice.

Cultivation of PBMC of patients with recurrence of disease on MEF

PBMC obtained from the three patients with recurrence of disease and from five control individuals were co-cultivated on MEF cultures. The results summarized in Table 2 show that the virus was activated from PBMC of all the three patients between 3 and 4 days of co-cultivation on fibroblasts. Of 15 cultures made from each patient only four or five were positive. The viruses isolated were identified as JEV by neutralization test in infant mice using hyperimmune JEV, WN and Dengue viruses sera (Table 3). No virus could be activated from JEV-seronegative control individuals.

Antibody response

IgM to JEV was determined by MAC ELISA in sera collected from patients during acute phase of illness from asymptomatic children 8 months after primary infection and from three children at the time of recurrent infection. The findings in Table 5 show that JEV IgM antibodies were present in all the acute sera of encephalitis patients. Two out of three sera collected from children who had recurrence of infection had IgM antibodies. No IgM antibodies were seen in sera of the asymptomatic and five normal children. The HAI findings in the paired sera from encephalitis patients revealed significant conversion of antibody titres with JEV. The antibody titres had fallen by 8 months period. A rise in JEV-specific HAI antibody titres was observed in the sera of the children who experienced recurrence of infections.

DISCUSSION

The salient feature of the present study is the observation that JEV established latent infection in peripheral blood mononuclear cells of children who had JEV infection 8–9 months previously. Some of these patients developed recurrence of disease despite the presence of circulating antibodies. Activated virus was isolated by co-cultivation from T lymphocytes of PBMC of asymptomatic children on MEF cells, by indirect immunofluorescence or by inoculation into the mice. The B lymphocytes and macrophages of latently infected children did not reactivate the virus.

JEV infection is one of the major causes of encephalitis world over with high mortality (Umenai *et al.*, 1985). Epidemics of JEV have been observed during October to December in post-monsoon season in Northern India since 1978 (Mathur *et al.*, 1982), and our recent observations suggest that JEV is probably endemic in this area (Kumar *et al.*, 1988). Edelman *et al.* (1976) reported persistence of the JEV-specific IgM antibody 116–135 days after acute illness and related it to acute virulence of JEV infection. There are studies describing the sequelae following JEV infection (Grossberg, Heyman & Keehn, 1962; Schneider *et al.*, 1974). To our knowledge, latency and recurrence of JEV infection in human have not been shown before.

JEV is a neurotropic virus. In fatal cases localization of viral antigen has been shown in the neurons (Johnson *et al.*, 1985). Attempts to isolate JEV in peripheral blood of acute JEV patients have been unsuccessful (Mathur *et al.*, 1982). We found that a small percentage of T lymphocytes and macrophages are infected in mice during acute infection (Mathur *et al.*, 1988). In the present study isolation of JEV from T lymphocytes of latently infected children has been documented. Successful activation of Epstein–Barr virus and HIV has been reported from various leucocyte subpopulations or macrophage/monocyte cells (Klein *et al.*, 1976; Harper *et al.*, 1986). Absence of JEV-specific IgM in blood of asymptomatic children supports the findings that virus has been truly latent in T lymphocytes. Monoclonal antibody studies are underway to establish that the JE serotypes recovered from patients' lymphocyte are identical to strains isolated during the JEV epidemic in Utar Pradesh, India, in 1978.

We have described the development of recurrence of symptoms due to latent JEV infections in three children. No virus was isolated during the acute stage of illness, while lymphocyte co-cultures were positive at the time of recurrence in these children. Patients with recurrence of JEV infection develop diminished lymphoproliferative response (manuscript in preparation). Currently the role of host responses in reappearance of disease in latent JEV infection is not clear. A study is underway to find out

the role of infection with other infectious agents with regard to activation of JEV infection. The phenomenon of latency and reactivation of virus continues to be a perplexing and challenging problem as its mechanism remains obscure. In mouse experiments the immunosuppression by cyclophosphamide or during pregnancy after latency resulted in the reappearance of JEV (Mathur *et al.*, 1986a) with the generation of quick, short-lived secondary immune response (Mathur, Kulshreshtha & Chaturvedi, 1987). The latently infected mice elicited diminished cell-mediated immune response as compared to control (Mathur *et al.*, 1989) and similar effects of JEV infection have been observed in asymptomatic, congenitally infected baby mice (Mathur *et al.*, 1986b). Some of the JEV-infected mice showed runting or developed hydrocephalus or died (unpublished data). Production of low molecular weight inhibitory factor by HSV-stimulated PBL has been shown (Sheridan *et al.*, 1987). This might help the virus to replicate sufficiently to cause clinical symptoms. The observations described here were made in only a few patients, and the results, therefore, require confirmation in larger series of patients.

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