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## Evidence for Latency of Japanese Encephalitis Virus in T Lymphocytes

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### SUMMARY

Activation of latent Japanese encephalitis virus (JEV) in the spleen has been studied by co-cultivation with allogeneic or syngeneic cells. Activated virus was isolated by co-cultivation from T lymphocytes of spleen, as shown by indirect immunofluorescence or by inoculation into mice. The B lymphocytes and macrophages of latently infected mice did not reactivate the virus. A higher proportion of Lyt 1 cells than Lyt 2 cells were harbouring JEV as shown by indirect immunofluorescence. The spleen cells from latently infected mice elicited the lymphoproliferative response but this was much lower than that observed in the controls. These findings suggest the establishment of latent JEV infection in T lymphocytes.

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Following primary infection of a host with certain viruses, a lifelong relationship can be established. Latent infection can be established in macrophages by cytomegalovirus (Brautigam *et al.*, 1979), in T lymphocytes by human immunodeficiency virus (Harper *et al.*, 1986), in B lymphocytes by Epstein-Barr virus (Klein *et al.*, 1976) and in sensory ganglion and mouse footpad cells by herpes simplex virus (Cook *et al.*, 1974; Clements & Subak-Sharpe, 1988). Japanese encephalitis virus (JEV) has the ability to establish a latent infection in mice after intraperitoneal (i.p.) inoculation (Mathur *et al.*, 1986*a*). The latent JEV infection is relatively silent in mice although it is associated with a defect in the cell-mediated immune response (Mathur *et al.*, 1986*b*). This characteristic is best demonstrated in patients who develop sequelae after JEV infection (A. Mathur, unpublished data). Reactivation of latent JEV in mice can be triggered by immunosuppression induced *in vivo* by cyclophosphamide or by pregnancy (Mathur *et al.*, 1986*a*). We have observed that JEV can establish latency within different organs of mice despite the presence of antiviral antibodies (Mathur *et al.*, 1986*a*), but the cellular sites of JEV latency have not been defined. Here we extend our previous observations in order to determine the cells that harbour virus and help to maintain JEV latency.

JEV strain 78668A was used as an adult mouse brain suspension (Mathur *et al.*, 1986*a*). The virus was propagated by intracerebral (i.c.) inoculation of mice and stored at  $-70^{\circ}\text{C}$ . The infectivity titre of the third passage pool in mice was  $10^{4.5}$  LD<sub>50</sub>. Inbred Swiss albino mice obtained from the mouse colony of this department were used throughout the study. Latent infection was established by i.p. inoculation of 6-week-old Swiss albino mice with 0.3 ml of 10% (w/v) JEV-infected mouse brain suspension. Animals were sacrificed at weekly intervals and organ (thymus, spleen, brain and kidney) homogenates (10%, w/v) were titrated for the presence of virus by i.c. inoculation into infant mice. No virus was detectable in any organ by 5 weeks, as has previously been observed (Mathur *et al.*, 1986*a*). These mice were kept under observation and were sacrificed 27 weeks later. The organs were collected, cut longitudinally and imprint smears were prepared on chilled slides, fixed with acetone and examined by indirect immunofluorescence as described earlier (Mathur *et al.*, 1988). The anti-JEV monoclonal antibody was kindly provided by Dr E. A. Gould (Arbovirus Research Unit, London School of Hygiene and Tropical Medicine, St Albans, U.K.) The organ homogenates were also assayed for the presence of virus by i.c. inoculation into infant mice. Mice that were shown to have JEV in

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Table 1. Recovery of JEV from different tissues of latently infected mice

Tissue	Time of recovery after activation (days)	JEV recovery after co-culture with MEF from mice*		Control
		Allogeneic	Syngeneic	
Thymus	10	6/11†	2/11	0/11
Spleen	12	5/11	2/11	0/11
Kidney	13	4/11	2/11	0/11

\* Cells ( $10^6$ ) from different organs of latently infected Swiss albino mice were co-cultured with MEF ( $10^5$ ) of Swiss albino and Parks strains of mice.

† Number of cultures from which JEV was recovered/total number.

any organ (2.1%) were excluded from the study, and the remainder were defined as latently infected.

We first studied the activation of latent JEV *in vitro* by using the co-cultivation method. Twenty-seven weeks after inoculation of JEV into Swiss albino mice, thymus, spleen and kidney tissues were co-cultivated with syngeneic or allogeneic mouse embryo fibroblasts (MEF); Parks strain mice were purchased from the Central Drug Research Institute, Lucknow. One half of each organ was homogenized (10%, w/v) and inoculated i.c. into infant mice after making imprint smears, which were examined by indirect immunofluorescence. The other half of the tissues, collected aseptically, were teased out into chilled Eagle's MEM containing 10% foetal calf serum, glutamine and antibiotics. Single-cell suspensions were prepared. Approx.  $10^6$  cells/culture were co-cultivated with syngeneic or allogeneic MEF ( $10^5$  cells/culture) in MEM-HEPES containing 10% foetal calf serum, glutamine,  $5 \times 10^{-5}$  M-2-mercaptoethanol and antibiotics. The cultures were incubated at 37 °C in the presence of 5% CO<sub>2</sub> and screened on alternate days for presence of JEV antigen.

Table 1 shows the rates of virus recovery from different organs of latently infected and control groups of mice. Six of the 11 latently infected thymic tissues released virus on allogeneic culture while two of 11 tissues yielded JEV on syngeneic culture. The findings of the present study show that the activation rate of JEV was markedly increased by using allogeneic co-culture, although this was not a prerequisite for activation of virus. Both syngeneic and allogeneic cells have been used in co-cultivation experiments for activation of latent virus (Olding *et al.*, 1975; Mayo *et al.*, 1978; Jordan *et al.*, 1982). The advantage of using the allogeneic cells in such experiments is the induction of blast cells, which are more conducive to virus replication. JEV was isolated most commonly between 10 to 17 days after co-cultivation in allogeneic or syngeneic culture, after which there was a decrease in isolation rate; no isolations were made after 22 days. The mean time of virus detection from the thymus was shortest. No virus was detected in the control groups of mice.

Further studies were carried out to determine the cell type responsible for harbouring the latent JEV. Cell populations enriched for T and B lymphocytes and glass-adherent cells were obtained from 27 week latently infected mice as described previously (Mathur *et al.*, 1983). The individual cell populations were co-cultivated with syngeneic and allogeneic mouse embryo cells and screened for activated virus. Briefly, a single cell suspension of spleen was prepared, the macrophage-enriched population was obtained by collecting the glass-adherent cells after layering the splenic cell suspension on glass Petri dishes for 2 h at 37 °C in the presence of CO<sub>2</sub>. More than 92% of the glass-adherent cells were phagocytic and were considered to be macrophages. The T and B cells were purified by filtration of the non-adherent cells through a nylon wool column using the technique of Julius *et al.* (1973). In different preparations 90 to 95% of cells were viable, as determined by the trypan blue dye exclusion test as described earlier (Mathur *et al.*, 1983). The purity was checked by treating these cells with anti-Thy1.2 antisera or anti-mouse IgG antisera (New England Nuclear) and complement as described before (Mathur *et al.*, 1984). The individual cell populations were co-cultivated on allogeneic MEF and screened for activated virus. Co-cultures were prepared by adding allogeneic MEF ( $10^5$  cells) to each cell

Table 2. Recovery of JEV from spleen cells of latently infected mice

Spleen cell subpopulations	Time of recovery after activation (days)	JEV recovery after co-culture with MEF*	
		Latently infected	Control
T lymphocytes	10	10/18†	0/18
B lymphocytes	0	0/18	0/18
Glass-adherent cells	0	0/18	0/18

\* Virus identified as JEV by immunofluorescence and by i.c. inoculation into infant mice and confirmation by neutralization test.

† Number of cultures from which JEV was recovered/total number.

subpopulation ( $10^6$  cells per culture) separately. Table 2 shows the data obtained after co-cultivation of cells from latently infected mice on allogeneic MEF for various periods of time. The results indicated that in 10 of 18 (55%) mice virus was recovered from T cells after co-cultivation. The virus was never detected before the 10th day of co-cultivation. Fifteen days after infection only 13% of the T cells were immunofluorescence-positive. The B lymphocytes and macrophages from latently infected and uninfected control mice yielded no virus by i.c. inoculation of infant mice, and were immunofluorescence-negative.

The results show the activation of JEV in T lymphocytes of latently infected mice, thus suggesting that the T lymphocytes can be a virus reservoir. Our earlier observations showed isolation of JEV from T cells and macrophages after i.p. inoculation of virus suggesting that the spread of viral infection is probably mediated by these cells (Mathur *et al.*, 1988). These data indicate that JEV, in addition to its well known neurotropism, might also have lymphotropic properties. In other studies, in which different cell populations in latent viral infections have been examined, lymphocytes have been found to be the predominant cell type harbouring the virus. Olding *et al.* (1975) have demonstrated that mouse cytomegalovirus establishes a latent infection in small sets of lymphocytes and macrophages and can be activated by co-cultivation of lymphocytes with allogeneic cells. Human cytomegalovirus RNA has been shown in lymphocytes of naturally infected individuals (Schrier *et al.*, 1985).

Further, we defined the subpopulation of T cells that harbours JEV in a latent state. The T lymphocyte enriched subpopulation of spleen cells was obtained from mice that had been inoculated with JEV 27 weeks earlier. Lyt 1- or Lyt 2-depleted cells were prepared from T cells by specific complement-mediated lysis with monoclonal antibodies against these subpopulations as described by Eskola *et al.* (1983). Specificity of depletion treatment was checked by an indirect immunofluorescence assay. The population was more than 92% pure. The anti-Lyt 1 and anti-Lyt 2 fluorescein-conjugated and native antibodies were purchased from Becton Dickinson).

The Lyt 1- and Lyt 2-enriched subpopulations were co-cultivated on allogeneic MEF cultures. Results after co-cultivation of different subpopulations of T cells indicated that about 17% of Lyt 1<sup>+</sup> cells and 2% of Lyt 2<sup>+</sup> cells expressed JEV antigen as examined by indirect immunofluorescence. It is thus documented that latent JEV established itself in both helper and suppressor phenotypes of T cells, though a higher percentage was found in Lyt 1 cells responsible for helper function. These findings demonstrate the selectivity of JEV for maintenance of latency and explain the activation of JEV from different organs in latently infected mice (Mathur *et al.*, 1986a).

Subsequently, experiments were carried out to study the lymphoproliferative response to JEV in latently infected mice. The splenic cells ( $2 \times 10^6$  cells/ml) from these mice in HEPES buffer, containing 5% foetal calf serum and antibiotics, were stimulated with 1  $\mu$ g/ml phytohaemagglutinin (PHA) (Burroughs-Wellcome) for 48 h at 37 °C in 5% CO<sub>2</sub>; [<sup>3</sup>H]thymidine was added for the last 4 h of culture. The assay was performed in triplicate. The findings, given in Table 3, show that the lymphoproliferative response was significantly depressed in latently infected mice as compared to controls ( $P < 0.01$ ), indicating an altered cell-mediated immune response in

Table 3. Comparison of spleen proliferative response of latently infected mice with controls

Spleen cells* from groups of mice	PHA-induced [ <sup>3</sup> H]thymidine incorporation† (c.p.m.)
Control	15531 ± 1023
Acutely JEV-infected	21356 ± 1123
Latently JEV-infected	8232 ± 936

\* Spleen cells from control mice, mice infected i.p. (7 days post-infection) or latently infected mice (27 weeks post-infection) were stimulated with PHA and labelled with [<sup>3</sup>H]thymidine for the last 4 h.

† Results are expressed as the mean from three PHA-stimulated cultures minus mean values from unstimulated cultures (± standard deviation).

latently infected mice. Therefore, the suppressed lymphoproliferative response to JEV could be considered either due to alterations in T lymphocyte numbers, to phenotypic changes in T lymphocyte subpopulations or to interference with T cell function by the presence of the viral genome in lymphocytes of latently infected mice.

To determine the T cell profile in the spleens of latently infected and control mice the spleen cells were treated with anti-Thy1.2 antibodies and complement. No significant difference in the percentage of T cells was found between the two groups of mice. Thus a deficiency of T cells was excluded. We found an altered helper:suppressor cell ratio in latently infected mice (data not shown). The possibility of incorporation of the JEV genome into helper cells which might interfere with their proliferation and function has to be studied by using *in situ* hybridization with JEV-specific probes.

Shiraki (1970) postulated JEV persistence in the brains of three patients who died 8 to 15 years after the onset of Japanese encephalitis. A parallel situation of latent JEV infection in lymphocytes has been recently found in man (A. Mathur, unpublished data).

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