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Induction of secondary immune response by reactivated Japanese encephalitis virus in latently infected mice

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SUMMARY

Development of secondary immune response has been studied following reactivation of latent Japanese encephalitis virus (JEV) infection in mice. The virus could be reactivated in 43% of the latently infected mice at 27 weeks p.i. by treatment with cyclophosphamide. The reactivated virus induced delayed-type hypersensitivity (DTH) and leucocyte migration inhibition (LMI) responses in mice, with peak activity on Day 5 post-reactivation (p.r.). The DTH persisted at low levels for long periods. Humoral immunity measured by haemagglutination-inhibiting antibody showed a four-fold rise in antibody titres. DTH was transferable by immune spleen cells for 5 days p.r. only. It is, therefore, concluded that JEV reactivation generates a quick and short-lived secondary immune response.

INTRODUCTION

A primary infection of adult Swiss albino mice with Japanese encephalitis virus (JEV) results in the development of short-lived protective cell-mediated and humoral virus-specific immune responses (Mathur, Arora & Chaturvedi, 1983a). These responses decline after 2 weeks of infection with the appearance of antigen-specific suppressor T cells (Mathur, Rawat & Chaturvedi, 1984). JEV has the ability to establish persistent infection in animals or in cell cultures (Mathur *et al.*, 1986a; Schmaljohn & Blair, 1979). The virus persists in a latent state *in vivo* and can be reactivated by inducing immunosuppression either by pregnancy or by treatment with cyclophosphamide (Mathur *et al.*, 1986a).

An optimum priming with an antigen induces formation of a pool of specifically prepared T and B memory cells (Miller & Sprent, 1971). The precursors of memory cells respond in an accelerated way to further exposure to the antigen, resulting in the secondary immune response. Slight increase in secondary immune response as a carry-over from the primary response has been demonstrated in lactic dehydrogenase virus infection (Isakove, Feldman & Segal, 1982). In the present study we have investigated the effect of reactivation of JEV on immunologically committed lymphoid cells in adult mice. We observed that virus reactivation provides a stimulus for quick generation of effector immune response. The secondary immune response lasts for a brief period of time.

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MATERIALS AND METHODS

Antigen

Japanese encephalitis virus (JEV), strain 78668A, isolated from human brain was propagated in adult Swiss albino mouse brain and stored at −70°, The virus was used as a 10% suspension in chilled Eagle's minimum essential medium (MEM). The infectivity titre of three-times passaged brain pool in infant mice was $10^{5.2}$ LD_{50}.

Infection of mice with JEV

Inbred Swiss albino mice (obtained from this Department) were inoculated with cyclophosphamide (CY-Endoxon ASTA, Khandelwal Lab. Pvt. Ltd, ASTA Werke, Bielefeld, FRG) in doses of 200 mg/kg body weight. A single intraperitoneal (i.p.) injection of 10% (w/v) JEV-infected brain suspension was given 4 days later. The mice were monitored daily for 2 weeks and then twice per week thereafter. Mice showed no morbidity or mortality.

Reactivation of virus

For reactivation of virus, 27-week latently JEV-infected mice were inoculated with 200 mg/kg body weight of cyclophosphamide (CY) intraperitoneally (Mathur *et al.*, 1986a). For the study of primary response, normal mice received CY and, 4 days later, JEV.

Virus isolation

Isolation of JEV from 10% tissue homogenate (thymus) has been described in detail previously (Mathur *et al.*, 1983a).

Antibody estimation

Blood samples were collected repeatedly at intervals from the

same mice by cutting the tail end. JEV-specific haemagglutination-inhibition (HAI) antibodies were estimated by the technique of Clarke & Casals (1958) using a microtitre technique as described elsewhere (Mathur *et al.*, 1983a). The antigen used was supplied by the Director, National Institute of Virology, Pune, India.

T-cell depletion of spleen cells

The T cells were depleted from spleen cell suspension by treatment with anti-Thy 1.2 antibodies (New England Nuclear, Boston, MA) and guinea-pig complement as described previously (Mathur *et al.*, 1984).

Leucocyte migration inhibition test

The previously described (Mathur *et al.*, 1983a), modified technique of David *et al.* (1964) was used. The test from each mouse was done in triplicate. The percentage migration inhibition was calculated by the following formula:

$$\% \text{ inhibition} = \frac{\text{migration in presence of antigen}}{\text{migration in absence of antigen}} \times 100.$$

An inhibition of 20% or more was considered significant.

Adoptive transfer of immunity

Groups of syngeneic recipient mice primed with JEV i.p. 48 hr earlier were injected i.v. with spleen cells from immunized donors. The 24 hr footpad reaction on Day 7 was recorded.

Assay for DTH

Different groups of mice were primed with 10% JEV-infected mouse brain suspension. The mice were challenged in the hind footpads subcutaneously (s.c.) with an eliciting dose of 0·02 ml of 10^2 LD_{50} of JEV. The 24 hr increase in footpad swelling was measured on Day 7 using a dial calliper as described elsewhere (Mathur *et al.*, 1984). The results were expressed as the percentage of footpad increase at 24 hr. The specific DTH represents the reading after the subtraction of background footpad increase induced by the eliciting antigen in control mice.

RESULTS

Mice latently infected with JEV were treated with CY at 27 weeks to reactivate the virus, and their secondary humoral and cell-mediated immune responses were assessed. The day of reactivation of virus was considered as Day 0.

Period of virus persistence

Thymus tissue obtained at weekly intervals up to 5 weeks from different groups of mice were assayed for infective virus by i.c. inoculation into infant mice. After CY treatment of latently infected mice at 27 weeks p.i. 20% of mice on Day 3 and 23% of mice on Day 4 showed reactivation of virus. The findings presented in Table 1 show that the virus could be isolated for up to 2 weeks following reactivation. The period of virus shedding was significantly shorter than that of primary JEV infection (4 weeks). No virus was isolated from control 27-week latently infected mice given phosphate-buffered saline in place of CY and normal mouse brain suspension in place of the virus 4 days later.

Table 1. Isolation of virus from thymus after primary infection and reactivated group of mice

Time after infection (weeks)	Thymus			
	Infection in naive mice	Virus titre	Reactivation after 27 weeks	Virus titre (LD_{50})
1	+	$10^{1.5}$	+	$10^{1.5}$
2	+	$10^{2.6}$	+	$10^{0.9}$
3	+	$10^{2.0}$	−	—
4	+	$10^{0.9}$	−	—
5	−	—	−	—

DTH response

The primary and secondary DTH response measured by the increase in footpad swelling is shown in Fig. 1. The mice of both JEV-primed and virus reactivated groups developed a DTH response but differed in the time of appearance and magnitude of response. The secondary DTH response was maximum on Day 5, and then declined quickly with a low level of response persisting for up to 4 weeks. In JEV-primed mice the primary response was significantly higher and prolonged, as described earlier (Mathur *et al.*, 1984). Control latently infected mice showed an insignificant level of DTH response.

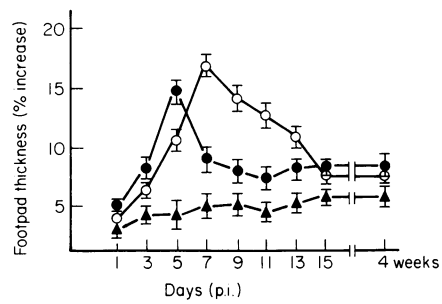

Figure 1. Kinetics of induction of secondary compared with primary delayed-type hypersensitivity response. Normal mice were inoculated with CY i.p. and primed with JEV 4 days later (○), and 27-week latently infected mice were challenged with CY (●); control latently infected mice were challenged with normal mouse brain suspension (▲). Each point represents the mean ± SD of five to seven mice.

Leucocyte migration inhibition response

The findings summarized in Fig. 2 show that the magnitude of LMI in the virus-reactivated group of mice was similar to that obtained with the primary response. The peak secondary LMI response was observed on Day 5, declined sharply by Day 7 and was insignificant after Day 9, whereas a significant LMI response was observed from Days 5 to 15, with a peak on Day 9 during the primary response. In the control mice the response was insignificant throughout the study period.

Adoptive transfer of DTH response

Spleen cells of the JEV-primed and reactivated group of mice

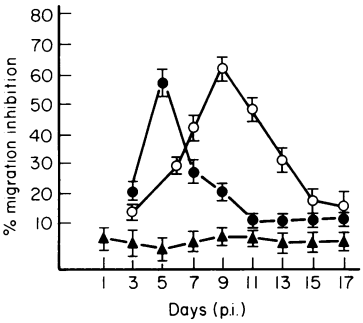

Figure 2. Comparison of leucocyte migration inhibition (LMI) responses to JEV after primary and secondary infections. The LMI of spleen cells was measured on alternate days. Each value represents the mean ± SD of specific antigen from triplicate experiments on cells from three to five mice. Normal mice were inoculated with CY i.p. and primed with JEV 4 days later (○), and 27-week latently infected mice were challenged with CY (●); control latently infected mice were challenged with normal mouse brain suspension (▲).

Table 2. Antibody response in mice at different periods after primary infection with JEV and after reactivation of virus

Time after infection (weeks)	JEV HAI* titre	
	Infection in naive mice	Reactivation after 27 weeks of infection
1	13 ± 1	80 ± 0
2	37 ± 2·1	178 ± 4·2
3	96 ± 3·7	72 ± 3·7
4	96 ± 2·0	—
27	43 ± 3	—

* HAI, haemagglutination-inhibiting antibody. The reciprocal dilution of serum giving inhibition against 8 HA units of antigen is presented. Each HAI value represents the mean ± SD from seven to nine mice.

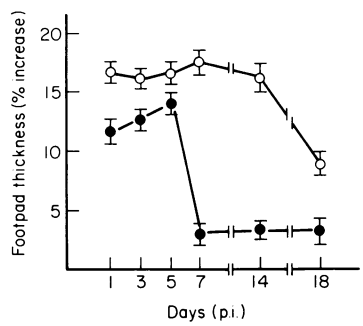

Figure 3. Effect of spleen cell transfer on delayed-type hypersensitivity response. Spleen cells from different groups of mice were collected and transferred (10^8 cells/0·25 ml) i.v. in recipients. Cells were transferred from normal mice primed with JEV (○), or from the virus-reactivated group of mice (●). DTH was elicited on Day 7. Each value represents the mean ± SD from three to five mice.

were collected on alternate days. The DTH response on adoptive transfer of cells in mice was studied. The findings summarized in Fig. 3 show that the secondary DTH response was transferred up to 5 days only after virus reactivation in mice. In contrast to this, the primary response could be transferred up to 17 days post-infection.

T-cell count

Spleens obtained at weekly intervals after primary JEV infection or reactivation of virus in mice were teased out and treated with anti-Thy 1.2 antibodies and complement. The non-viable cells were considered as T cells and expressed as a percentage of spleen cells. No significant difference in the percentage of T cells was found in the two groups of mice at any period.

Antibody response

The sera from mice at 1–4 weeks after primary infection or reactivation of the virus were assayed for JEV-specific haemagglutination-inhibiting (HAI) antibody. The sera from normal mice and those collected from latently infected mice just prior to reactivation of the virus were used as controls. All the sera were negative before priming with JEV and become positive for HAI antibody to JEV after primary infection (Table 2). However, the antibody titres had fallen by 27 weeks before reactivation. A four-fold rise in antibody titres was observed in mice that experienced reactivation of virus (Table 2).

DISCUSSION

We have observed earlier that latent JEV infection develops following a single i.p. dose of the virus to adult mice or following congenital infection when mother mice were infected during pregnancy (Mathur *et al.*, 1986a, b). The virus can be reactivated in such mice by inducing pregnancy or by CY treatment (Mathur *et al.*, 1986a, b). The results presented here demonstrate the development of secondary cell-mediated and humoral immune responses as a consequence of reactivation of the virus in latently JEV-infected mice. The virus could be reactivated in 43% of mice, which confirms our earlier observations (Mathur *et al.*, 1986a). Latency is predominantly a static phenomenon that would not be disturbed under normal conditions but, once reactivation of virus occurs, it provides opportunities for fresh cells to become infected. In the present study the virus was isolated from thymus for 2 weeks only. This brief period of virus detection could be due to the prompt development of the immune response. In comparison, in primary JEV infection the virus was isolated for 4 weeks p.i. The factors that modulate virus shedding are not known but probably the immunosuppression facilitates it. Experiments are under way to study whether the mice eliminates the virus or whether it becomes latent again.

A full range of humoral and CMI responses develop following primary JEV infection (Mathur *et al.*, 1983a). The secondary immune response to an antigen is characterized by a faster and more vigorous immune response; the humoral response is mainly by IgG rather than IgM antibodies, and the antibody titre may be up to 10- to 50-fold higher (Weir, 1983). A

comparison of the kinetics of primary and secondary immune responses to JEV shows that peak DTH and LMI responses developed earlier in the secondary response (Figs 1 and 2). The adoptive transfer of DTH in recipient mice was possible up to 5 days after reactivation and waned thereafter, in contrast to primary infection where the DTH could be transferred up to 17 days after infection. The finding that the reactivation of virus is associated with a brief and faster generation of CMI suggests the presence of T memory cells, but CMI lasts for a few days only. The rise in antibody titres was for 2 weeks and was only four-fold higher than the basal level. Studies are being undertaken to determine the nature of the antibodies that appeared after reactivation of the virus.

The differences observed in the present study from the classical secondary response merit discussion. Could the single dose of CY, an immunosuppressive drug, used for reactivation of the virus be responsible? CY has been shown to depress humoral immune response if given 48 hr before or after an antigen (Katz *et al.*, 1974; Rager-Zisman & Allison, 1976; Chaturvedi, Tandon & Mathur, 1977). CY given at a space of 96 hr has little effect on the immune response to the agent. In the present study the virus appears after about 96 hr, and the immune response develops after 120–144 hr of the drug treatment; therefore, CY should not materially affect the immune response.

The brief secondary immune response could be due to a deficiency of T cells, impaired antigen presentation, or activation of suppressor T cells. In JEV-infected mice, T-cell deficiency occurs in congenitally infected baby mice and not in adult mice (Mathur, Arora & Chaturvedi, 1983b). The reactivated JEV is present in very low titres. As the secondary immune response is dose dependent, this could well influence the outcome. Our recent findings, reported elsewhere, show the presence of memory suppressor T cells in mice latently infected with JEV. The reactivation of the virus results in the quick generation of suppressor T cells both for CMI (unpublished data) and for humoral response (Mathur *et al.*, 1987). These factors, singly or in combination, may be the cause of the lower secondary immune response in JEV-infected mice.

We have observed that, in spite of the development of both CMI and humoral immune response, the JEV persists in the host (Mathur *et al.*, 1986a). At the time of reactivation in latently infected mice, the virus provides the stimulus for the generation of the secondary immune response. The efficiency and speed of generation of the immune response from a state of immune memory help in the elimination of the virus. Further, the appearance of the suppressor cells temporarily favours the replication of virus. Thus, a low dose of antigen, a poor secondary immune response and suppressor cells will determine the outcome of virus. Edelman *et al.* (1976) have reviewed the reports suggesting the persistence of JEV in human cases, on the basis of various evidence, including the persistence of IgM antibodies. It is likely that a situation similar to that of the mouse model described by us exists in human cases, leading to persistence and latency of the virus. Studies are underway to find out whether the virus persists further and can be reactivated again.

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