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Persistence, Latency and Reactivation of Japanese Encephalitis Virus Infection in Mice

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SUMMARY

Persistent and latent Japanese encephalitis virus (JEV) infection was studied in pregnant and non-pregnant mice. Following intraperitoneal inoculation into pregnant mice JEV persisted for 16 weeks in contrast to 4 weeks in non-pregnant mice. This was followed by a higher frequency of latent infection in pregnant mice. The virus could be reactivated during pregnancy or by cyclophosphamide treatment, the latter being more effective.

Following widespread epidemics, Japanese encephalitis virus (JEV) has become endemic in different parts of India, with a sporadic occurrence of cases throughout the year (Indian Council of Medical Research, 1980). Despite extensive studies on non-human amplifiers and reservoirs, the mechanism of overwintering of JEV is still not clear. JEV usually causes acute illness but at times it may run a protracted course with acute exacerbations. In such cases the brain tissue shows chronic as well as acute lesions (Shiraki, 1970). It is not known if these are due to re-infection, or to reactivation of a persistent virus. Evidence of persistent infection with JEV has been described in mammalian and mosquito cell lines (Schmaljohn & Blair, 1979; Rehacek, 1968) and from studies in bats (Ito & Saito, 1952). Defective interfering particles have been found to be responsible for establishment and maintenance of JEV persistent infection *in vitro* (Schmaljohn & Blair, 1979).

A number of viruses can establish persistent infection in the host (Stroop & Baringer, 1982) but the mechanism of virus persistence is complex. The main feature in such an infection is evasion of the host immune response resulting in a failure to eliminate the virus (Mahy, 1985). We have described a JEV–mouse model to study the transplacental transmission of the virus (Mathur *et al.*, 1981), a phenomenon observed in human cases during the 1978 epidemic in India (Chaturvedi *et al.*, 1980). Pregnant mice initially infected during pregnancy could transmit JEV to foetuses 6 months later during subsequent pregnancies (Mathur *et al.*, 1982). These observations are of great significance if true for humans also. Therefore, in this report an effort was made to study the establishment of persistence, latency and reactivation of JEV in pregnant and non-pregnant mice.

JEV strain 78668A was used as an adult Swiss albino mouse brain suspension. Evidence has been presented earlier showing the presence of JEV in spleen, thymus and kidney up to 11 days after infection in mice inoculated during pregnancy (Mathur *et al.*, 1981). To examine further the period of virus production from different organs, 100 LD_{50} of JEV was given intraperitoneally (i.p.) to pregnant mice on day 8 of the pregnancy. Non-pregnant female mice of similar age were inoculated similarly. From each group six or seven mice were sacrificed at weekly intervals. Different organs, i.e. spleen, liver, kidney, thymus, ovary, brown fat and blood, were collected aseptically. Organ homogenates (10%, w/v) were assayed for the presence of JEV by intracerebral (i.c.) inoculation into suckling mice. Virus identification was done by

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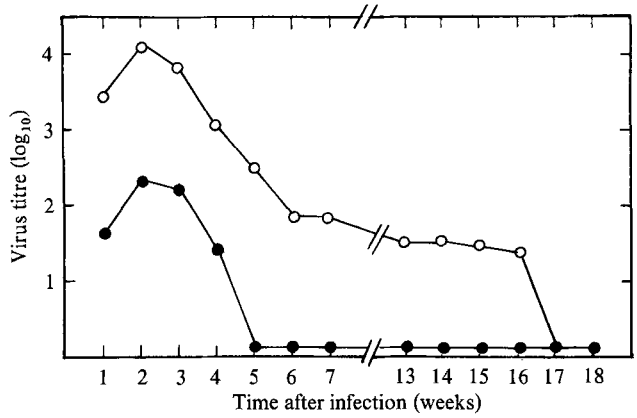

Fig. 1. Japanese encephalitis virus titres in the thymus of pregnant (○) and non-pregnant (●) mice inoculated i.p. with 10^2 LD_{50} virus. Each point represents the mean infectivity titre from six or seven mice individually assayed.

Table 1. *Isolation of virus from different organs of pregnant and non-pregnant mice given JEV by i.p. route*

Time after infection (weeks)	Positive isolations/total mice tested				
	Thymus	Spleen	Brown fat	Kidney	Ovary
1	6/6 (5/6)*	6/6 (5/6)	0/6 (0/6)	2/6 (4/6)	0/6 (0/6)
2	6/6 (3/6)	6/6 (3/6)	2/6 (3/6)	4/6 (3/6)	2/6 (2/6)
3	6/6 (3/6)	6/6 (3/6)	5/6 (2/6)	4/6 (2/6)	4/6 (1/6)
4	6/6 (2/7)	6/6 (0/7)	5/6 (2/7)	4/6 (0/7)	4/6 (0/7)
5	6/6	6/6	4/6	3/6	4/6
6	6/7	5/7	4/7	3/7	4/7
7	5/7	5/7	4/7	3/7	3/7
8	4/7	4/7	4/7	2/7	3/7
9	4/7	3/7	3/7	2/7	3/7
10	3/6	4/6	3/6	2/6	3/6
11	3/6	3/6	3/6	0/6	2/6
12	3/6	2/6	2/6	0/6	2/6
13	2/6	2/6	2/6	0/6	1/6
14	2/6	1/6	2/6	0/6	0/6
15	2/6	0/6	2/6	0/6	0/6
16	2/6	0/6	1/6	0/6	0/6

* Results for non-pregnant mice are given in parentheses.

neutralization tests in infant mice using hyperimmune sera (Mathur *et al.*, 1981) supplied by the National Institute of Virology, Pune, India.

The results of virus isolation from different organs of pregnant mice at various times after i.p. infection are summarized in Table 1. The virus was isolated from thymus and spleen in all mice tested 1 to 5 weeks after infection. Of the 100 mice examined over a period of 16 weeks, JEV was consistently recovered from all organs shown in Table 1. The longest periods during which virus could be isolated (up to 16 weeks) were observed in thymus and brown fat. Blood and liver contained virus in the first week only, and no virus could be isolated from brain tissue on any occasion. Virus titres in the thymus reached $10^{4.3}$ LD_{50}/g of tissue at the second week, which was higher than at any other time and in any other organ (Fig. 1).

The findings summarized in Table 1 show a briefer period of virus shedding after JEV infection of non-pregnant mice than of the pregnant mice. In this group also the JEV isolation rate was higher in the thymus (13/25) than in any other organ. Maximum infectivity titres in the

Table 2. *Antibody response after i.p. inoculation of JEV in pregnant and non-pregnant mice*

Time post-immunization (weeks)	JEV HAI titre in serum*			
	Pregnant		Non-pregnant	
	Buffer†	2-ME‡	Buffer†	2-ME‡
Before inoculation	<8	<8	<8	<8
1	12 ± 2·1	<8	11 ± 4·3	<8
2	51 ± 3	8	42 ± 3·5	8
3	102 ± 21	90 ± 7	90 ± 11	90 ± 11
4	102 ± 8	102 ± 15	102 ± 19	102 ± 4
5	102 ± 19	102 ± 9·1	102 ± 15	102 ± 15
27	90 ± 3·5	90 ± 3·2	90 ± 7	90 ± 12

* HAI, Haemagglutination inhibiting antibody. The reciprocal dilution of the serum giving inhibition against 8 HA units of antigen is presented. Each HAI value represents the mean value with S.D. from five to seven mice.
† Serum aliquot treated with buffer.
‡ Serum aliquot treated with 2-mercaptoethanol.

thymus were $10^{2\cdot1}$ to $10^{2\cdot5}$ LD_{50}/g of tissue at the second week, which was significantly less than that in pregnant mice (Fig. 1). No virus was isolated from brain at any time.

An attempt was made to isolate infectious virus between 17 and 26 weeks after infection from mice infected during pregnancy and 5 to 26 weeks post-infection from those infected when not pregnant. Every week three to five mice of each group were killed and tests for virus made on tissue homogenates (thymus, spleen, kidney, brain, brown fat and ovaries). Infectious virus was present in tissues of only one out of 45 (2·2%) mice in the pregnant group and in none of the 46 non-pregnant animals. From each of the remaining mice an open surgical biopsy of the thymus was done at week 26 and the resected tissue was homogenized and assayed for JEV. Mice that had JEV in the thymus were excluded from the study and the remainder were defined as latently infected.

For reactivation experiments those latently infected mice were selected which had shown evidence of infection after i.p. inoculation of virus either by *in utero* transmission of virus to foetuses or by the presence of JEV-specific haemagglutination-inhibiting (HAI) antibodies. After 27 weeks an attempt was made to reactivate the virus by inducing pregnancy (presence of vaginal plug after mating with normal male) or by cyclophosphamide (CY) treatment (200 mg/kg body weight, i.p.; Endoxan-ASTA, Khandelwal Laboratories Pvt., ASTA Werke AG, F.R.G.). On alternate days, seven to 11 mice from each group were sacrificed and thymus, spleen, liver, brown fat and blood tested for infectious virus.

After CY inoculation at 27 weeks post-infection, seven of 11 mice infected during pregnancy and five of 11 infected when not pregnant showed reactivation of virus. The virus was isolated in both groups from blood and thymus as early as on day 3 after CY treatment and later from other organs. Reactivation occurred during pregnancy in five of 11 mice originally infected when pregnant and in four of 11 mice originally infected when not pregnant. CY is a potent immunosuppressive agent and caused reactivation of the virus more frequently than did pregnancy. Some of the mice in each of the groups were allowed to give birth. Reactivated virus can be transmitted transplacentally, resulting in neonatal deaths as reported earlier (Mathur *et al.*, 1982). Virus was isolated from 13 to 17% of the newborns from these groups.

Reactivation of virus was also found in mice infected with JEV approximately 1 year earlier and found to be latently infected as defined above by thymic biopsy. After CY inoculation at 52 weeks after infection reactivation occurred in seven of 11 mice infected during pregnancy and four of nine infected when not pregnant. The virus was isolated from thymus and blood. This shows that virus can remain latent for at least 1 year.

The findings summarized in Table 2 show the HAI (estimated by the method of Clarke & Casals, 1958) and IgM (estimated before and after 2-mercaptoethanol treatment) antibody titres in serum before and after JEV infection. The peak HAI antibody titres were observed at 3 weeks post-infection in both infected groups, while the JEV IgM HAI antibodies were present for only

2 weeks after infection and were absent at 17 weeks in all the sera tested at 27 weeks after infection. The HAI antibody titres were maintained at approximately the same level. JEV evidently persisted in mice despite the continuous presence of significant levels of serum antibody.

Protection against JEV is provided by T lymphocytes and by IgM antibodies which are present until about 2 weeks after infection (Mathur *et al.*, 1983). At later times these responses are depressed due to the appearance of specific suppressor T cells (Mathur *et al.*, 1984). If the short-lived protective cell-mediated immunity and IgM antibody responses by themselves are not sufficient to terminate infection, the virus persists. Papovaviruses (Walker & Padgett, 1983) and herpes simplex viruses (Wildy & Gell, 1985) can persist throughout the life of the host in a latent form.

The virus titres were significantly higher in different organs of pregnant mice than in non-pregnant mice. Furthermore, the virus persisted for up to 4 weeks in non-pregnant mice after primary infection while in pregnant mice it persisted for 16 weeks. Pregnancy is known to be associated with immunosuppression. Therefore, failure of pregnant mice to clear JEV for up to 16 weeks might be a result of pregnancy-induced immunosuppression. Immunosuppression delays coxsackie B4 virus elimination from ICR Swiss mice (Khatib *et al.*, 1983) and may help in establishment of persistent infection of lymphocytes and macrophages by lentiviruses (Mims, 1982). An increase in virus titre due to immunosuppression after CY treatment has been reported in coxsackie B3 (Rager-Zisman & Allison, 1973) and dengue 2 (Chaturvedi *et al.*, 1977) virus infections of mice.

The simultaneous appearance of virus in thymus and blood before detection in other organs may indicate that it is reactivated in the thymus and then disseminated; the above experiments show that infectious JEV persists longer in the thymus than in other organs. Thymic cells might therefore be useful for the study of latency.

Attenuated JEV vaccines have been developed to control infection in humans as well as in JEV amplifiers. Live attenuated vaccines for bluetongue virus (BTV 10) and Rift Valley fever virus have been implicated in causing congenital disorders when administered during pregnancy (Shultz & DeLay, 1955; Coetzer & Barnard, 1977), and similar effects should be considered for JEV vaccines.

The natural route of JEV infection in man is through the bite of a mosquito, which cannot be equated with the i.p. route used in our mouse experiments. However, inapparent infection and transplacental transmission of the virus seen in human cases (Chaturvedi *et al.*, 1980; Mathur *et al.*, 1985) can be reproduced in mice by giving JEV i.p. (Mathur *et al.*, 1981). Findings on the persistence, latency and reactivation of JEV in the mouse model suggest that such phenomena could occur in human cases.

REFERENCES

CHATURVEDI, U. C., TANDON, P. & MATHUR, A. (1977). Effect of immunosuppression on dengue virus infection in mice. *Journal of General Virology* **36**, 449–458.

CHATURVEDI, U. C., MATHUR, A., CHANDRA, A., DAS, S. K., TANDON, H. O. & SINGH, U. K. (1980). Transplacental infection with Japanese encephalitis virus. *Journal of Infectious Diseases* **141**, 712–715.

CLARKE, D. H. & CASALS, J. (1958). Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. *American Journal of Tropical Medicine & Hygiene* **7**, 561–573.

COETZER, J. A. W. & BARNARD, B. J. H. (1977). Hydrops amnii in sheep associated with hydroencephaly and arthrogrypasis with Wesselsborn disease and Rift Valley fever viruses as aetiological agents. *Onderstepoort Journal of Veterinary Research* **44**, 119–126.

INDIAN COUNCIL OF MEDICAL RESEARCH (1980). *Japanese Encephalitis in India*, 2nd edn. New Delhi: Indian Council of Medical Research.

ITO, T. & SAITO, S. (1952). Susceptibility of bats to Japanese B encephalitis virus. *Japanese Journal of Bacteriology* **7**, 612–622 (in Japanese).

KHATIB, R., KHATIB, G., CHASON, J. L. & LERNER, A. M. (1983). Alteration in coxsackie virus B4 heart muscle disease in ICR Swiss mice by anti-thymocyte serum. *Journal of General Virology* **64**, 231–261.

MAHY, B. W. J. (1985). Strategies of virus persistence. *British Medical Bulletin* **41**, 50–55.

MATHUR, A., ARORA, K. L. & CHATURVEDI, U. C. (1981). Congenital infection of mice with Japanese encephalitis virus. *Infection and Immunity* **34**, 26–29.

MATHUR, A., ARORA, K. L. & CHATURVEDI, U. C. (1982). Transplacental Japanese encephalitis virus (JEV) infection in mice during consecutive pregnancies. *Journal of General Virology* **59**, 213–217.

MATHUR, A., ARORA, K. L. & CHATURVEDI, U. C. (1983). Host defence mechanisms against Japanese encephalitis virus infection in mice. *Journal of General Virology* **64**, 805–811.

MATHUR, A., RAWAT, S. & CHATURVEDI, U. C. (1984). Suppressor T cells for delayed type hypersensitivity to Japanese encephalitis virus. *Immunology* **52**, 395–402.

MATHUR, A., TANDON, H. O., MATHUR, K. R., SARKARI, N. B. S., SINGH, U. K. & CHATURVEDI, U. C. (1985). Japanese encephalitis virus infection during pregnancy. *Indian Journal of Medical Research* **81**, 9–12.

MIMS, C. A. (1982). Role of persistence in viral pathogenesis. In *Virus Persistence*, pp. 1–13. Edited by B. W. J. Mahy, A. C. Minson & G. K. Darby. Cambridge: Cambridge University Press.

RAGER-ZISMAN, B. & ALLISON, A. C. (1973). Effects of immunosuppression on Coxsackie B-3 virus infection in mice, and passive protection by circulating antibody. *Journal of General Virology* **19**, 339–351.

REHACEK, J. (1968). Persistent infection of mosquito cells grown in vitro with Murray valley encephalitis and Japanese encephalitis viruses. *Acta virologica* **12**, 340–346.

SCHMALJOHN, C. S. & BLAIR, C. D. (1979). Clonal analysis of mammalian cell cultures persistently infected with Japanese encephalitis virus. *Journal of Virology* **31**, 816–822.

SHIRAKI, H. (1970). Japanese encephalitis. In *Clinical Virology*, pp. 155–175. Edited by R. Debre & J. Celers. Philadelphia: W. B. Saunders.

SHULTZ, G. & DELAY, P. D. (1955). Losses in new born lambs associated with blue tongue vaccination of pregnant ewes. *Journal of the American Veterinary Medical Association* **127**, 224–226.

STROOP, W. G. & BARINGER, J. R. (1982). Persistent, slow and latent viral infections. *Progress in Medical Virology* **28**, 1–43.

WALKER, D. L. & PADGETT, B. L. (1983). Progressive multifocal leukoencephalopathy. In *Comprehensive Virology*, vol. 18, pp. 161–163. Edited by H. Fraenkel-Conrat & R. R. Wagner. New York: Plenum Press.

WILDY, P. & GELL, P. G. H. (1985). The host response to herpes simplex virus. *British Medical Bulletin* **41**, 86–91.

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