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Congenital Infection of Mice with Japanese Encephalitis Virus

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Transplacental transmission of Japanese encephalitis virus (JEV) when given intraperitoneally was demonstrated in pregnant mice as shown by isolation of the virus from placenta and fetal tissues. Furthermore, JEV could be isolated from the brain, liver, and spleen of newborn mice. The effect of JEV at different periods of gestation in pregnant mice was demonstrated for the first time, and the consequences of maternal infection on fetuses and neonates were studied. JEV infection during the 1st week of gestation caused a significantly higher number of fetal and neonatal deaths (66%) than during the 3rd week of gestation (13.8%). The number of abortions, stillbirths, and neonatal deaths was higher in infected mothers than in controls. No congenital abnormalities were found in any of the newborn mice. Sera obtained from 5-week-old healthy mice delivered by mothers infected during the 3rd week of gestation contained JEV hemagglutination inhibiting and immunoglobulin M antibodies. The results of these preliminary experiments show the usefulness of mice as a model for further elucidation of JEV infection during pregnancy and its effects on the fetus.

Although the teratogenic potential of Japanese encephalitis virus (JEV) in pregnant women is not known, Chaturvedi et al. (2) reported isolation of JEV from human placenta and fetuses during an epidemic in Uttar Pradesh, India, in 1978. In a subsequent epidemic in 1980, JEV was again isolated from placenta in cases of abortion (A. Mathur et al., unpublished data). The virus has caused many widespread epidemics in India involving a large section of the population (7), thus posing a threat to pregnant women in the area. Japanese workers have reported an increased incidence of stillbirths and deaths of newborn swine during a JEV epidemic and the isolation of JEV from the brain of a stillborn piglet (1, 8). The immune status of the mother and the gestation period at the time of infection are critical factors in adverse effects on the fetus. Before an effort is made to identify and protect patients at risk, it is essential to establish a consistent causal relationship of maternal infection to fetal death. A suitable animal experimental model would provide an ideal means to validate the clinical observations and also to study the various factors affecting transplacental transmission. We describe here an animal model of congenital infection with JEV.

MATERIALS AND METHODS

Virus. A JEV strain (78668A) isolated from human brain during a widespread JEV epidemic in India during 1970 was used throughout the study. Initial typing was done by a quick complement fixation test, and final identification was by a neutralization test against hyperimmune sera of JEV (Mathur et al., Indian J. Med. Res., in press). The virus was passed intracerebrally (i.c.) 8 to 10 times in adult Swiss albino mice and produced uniform sickness and 100% mortality by day 6. Clinical illness and mortality were not seen in adult mice when the virus was given intraperitoneally (i.p.). Infected mouse brain pools were stored at −70°C. The infectivity titers of the brain pools, measured by i.c. injection of serial 10-fold dilutions in adult mice, ranged from $10^{4.1}$ to $10^{5.3}$ 50% lethal doses (LD_{50}). The titers are expressed as log 10 per gram (wet weight) of organ.

Transmission experiments. Transplacental transmission of the virus was studied by inoculating 10^2 LD_{50} of JEV i.p. in mice on the 8th day of pregnancy. Localization of the virus in brain, liver, spleen, and placenta was studied on days 1, 2, 3, 4, 5, 7, 9, and 11 after inoculation. Blood from infected mice was collected at the same time. The tissues were collected aseptically on different days.

Assay of virus in different organs. Different tissues from three or more infected pregnant mice were collected at specified intervals. The tissues were chopped and repeatedly washed with cold Hanks balanced salt solution until they were free from blood. Fetal brain and carcasses with skin removed were assayed for viral content. Brain tissue was collected from stillborn mice, from newborn sick mice (within 24 h of delivery), and from some of the mice during the neonatal period (within 30 days of birth). Liver, spleen, and brain tissues were collected from one of the infants. Ten percent (wt/vol) organ suspensions were prepared in Eagle minimal essential medium with antibiotics plus 5% heat-inactivated newborn calf serum and centrifuged in the cold. One-day-old suckling mice were inoculated i.c. with serial 10-fold dilutions in minimal essential medium of tissues and blood.

Clinical symptoms and death were noted. Virus was identified by either a complement fixation test or a neutralization test, using hyperimmune sera against JEV provided by the director of the National Institute of Virology, Pune, India.

Effect of JEV at different periods of gestation. Five pregnant mice were inoculated i.p. with 10^2 LD_{50} of JEV on the 5th day of pregnancy; five more were inoculated on the 8th day, and another five were inoculated on the 15th day. Inoculated animals were carefully watched for abortions, stillbirths, live births, and neonatal deaths. The total numbers of mice born, including stillbirths, were noted. Neonatal deaths were recorded up to 1 month after birth. Comparable control pregnant mice, inoculated with an uninfected mouse brain suspension, were kept and studied in the same manner.

Study of developmental anomalies. In another set of pregnant mice, inoculated with JEV at the same periods of gestation as described above, developmental anomalies were studied. Four pregnant mice were included in each group. Two control pregnant mice from each group were inoculated with an uninfected brain suspension. Approximately 1 day before the expected date of delivery, these mice were anesthetised and exsanguinated. The uteri were removed and examined for evidence of fetal abnormalities and resorption.

Serological tests. Blood was collected from pregnant mice by cutting the tail end. Sera were examined for hemagglutination inhibition (HI) antibodies by the method of Clarke and Casals (3), using a sucrose acetone-extracted, freeze-dried antigen of JEV. The antigen was supplied by the director of the National Institute of Virology, Pune, India. Tests for specific immunoglobulin M (IgM) antibodies were made before and after treatment with 2-mercaptoethanol (4).

Statistical method. Data were statistically analyzed by the Student t test for P values.

RESULTS

Infection in pregnant mice. We wished to determine whether JEV crosses the placenta and infects the fetus in mice. Eight-day-old pregnant mice without JEV antibodies were inoculated i.p. with 10^2 LD_{50} of JEV. No clinical illness was observed in the inoculated mice. Figure 1 shows the distribution of the virus in blood, liver, spleen, kidney, and placenta on days 1, 2, 3, 4, 5, 7, 9 and 11. The virus was recovered from the blood on days 1 and 2 only. The virus was found in the liver on day 2, reached a peak on day 5, and became undetectable by day 7. Virus was first recovered from the spleen and placenta on day 4 and persisted throughout the study period.

Effect of JEV infection at different stages of gestation in mice. To determine the effect of JEV given at different gestation periods, mice without JEV antibodies were inoculated i.p. with 10^2 LD_{50} of JEV on day 5, 8, or 15 of pregnancy. In each group, five infected and three control mice were observed. Of five pregnant mice inoculated during the 1st week of gestation, one aborted and four gave birth prematurely, after 17 to 18 days of pregnancy, to a total of 21 offspring. Control pregnant mice delivered on day 19 or 20 of pregnancy. Among the 21 babies, 4 were stillborn (21%) and 10 died during the neonatal period (58%). There was a significant increase in stillbirths and neonatal deaths in these mice. Infected mice gave birth to significantly fewer offspring than did control mice ($P < 0.025$) (Table 1). No abortions were noted in the control group. Among the mice infected during the 2nd week of gestation, one aborted on day 14 of gestation and four delivered at term. The total number of infants delivered by infected mothers was 35, which included 8 stillbirths (23%) and 9 neonatal deaths (33%). Statistically significant ($P < 0.05$) differences were observed in the number of infants delivered by the control and infected groups (Table 1). In pregnant mice inoculated during the 3rd week of gestation, no evidence of abortions or stillbirths was found. Neonatal deaths were observed in 6 of a total of 43 mice born (13.8%) (Table 1).

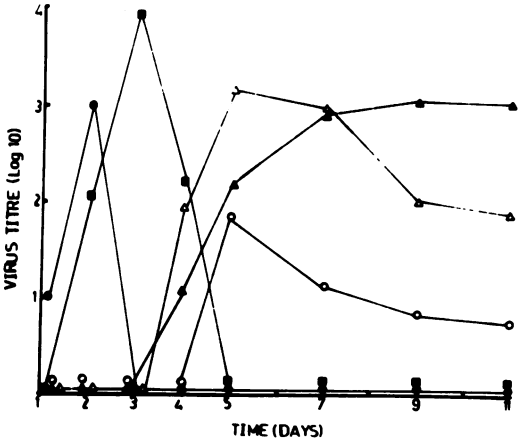

FIG. 1. *JEV titers in different organs of 8-day-old pregnant mice after intraperitoneal inoculation of virus. Symbols: ●, Blood; ■, liver; △, placenta; ▲, spleen; ○, kidney. Each point represents the mean value for three mice.*

Developmental abnormalities. Mice infected during the 1st week of pregnancy gave birth to significantly fewer offspring than did uninfected mice. To determine the developmental defect during gestation, 12 pregnant mice, 4 in each week of gestation, were inoculated i.p. with 10^2 LD_{50} of JEV. The pregnant mice were killed about 1 day before their expected date of delivery. The fetuses were counted and examined for any gross abnormalities. Mice inoculated on day 5 of gestation had 36 fetuses, of which 6 (17%) were undeveloped, 1 was resorbed,

TABLE 1. *Effects of maternal JEV infection on pregnancy at different stages of gestation*

Wk of gestation at inoculation	Group	No. of pregnant mice	No. aborted (%)	No. of stillbirths/ total no. delivered (%)	No. of neonatal deaths/total (%)	Total no. affected/ total no. delivered (%)
First	Virus	5	1 (20)	4/21 (21)	10/17 (58)	14/21 (66)
	Control	3	0 (0)	0/27 (0)	1/27 (3.7)	1/27 (3)
Second	Virus	5	1 (20)	8/35 (23)	9/27 (33)	17/35 (48.6)
	Control	3	0 (0)	0/28 (0)	2/28 (7)	2/28 (7)
Third	Virus	5	0 (0)	0/43 (0)	6/43 (13.8)	6/43 (13.8)
	Control	3	0 (0)	0/26 (0)	1/26 (3.8)	1/26 (3.8)

and 2 were dead. JEV was isolated from the undeveloped fetuses, whereas no virus was isolated from dead fetuses. Among the mice inoculated on day 8 of gestation, one fetus was dead. No gross abnormalities were observed in fetuses of mice inoculated on day 8 or 15 of gestation. In the control mice, no dead or resorbed fetuses were found.

Virus isolation from fetus and infants. Table 2 shows the results of virus isolation from fetuses and infants of pregnant mice given JEV at different periods of gestation. The virus was isolated from the brain of 1 of 4 stillborn mice, 6 of 9 newborn sick mice, and 5 of 15 mice during the neonatal period. The virus was isolated from the brain, liver, and spleen of one infant mouse.

Serological studies. Virus given during the 3rd week of gestation had little effect on fetuses or newborn infants, being rarely isolated from these mice. To examine the effect of fetal exposure to the virus, newborn infants were allowed to grow to the age of 5 weeks, at which time their sera were examined for HI and IgM antibodies to JEV (Table 3). Of nine mice examined, six had HI and virus-specific IgM antibodies (Table 3). No antibodies were detected in the sera of control mice.

TABLE 2. *Isolation of virus from infant mice*

Wk of gestation at inoculation	Group	Tissue of virus isolation	No. tested	No. from which virus was isolated	% Isolation
First	Stillborn	Brain	2	1	50.0
	Newborn (sick)	Brain	5	3	60.0
	Infant (neonatal period)	Brain	3	1	33.3
Second	Stillborn	Brain	2		0
	Newborn (sick)	Brain	4	3	75.0
	Infant (neonatal period)		5	2	40.0
	Infant	Brain	1	1	
		Liver	1	1	
		Spleen	1	1	
Third	Infant (neonatal period)	Brain	7	2	29.0

DISCUSSION

This study demonstrates that an active infection occurred in pregnant mice given JEV by the i.p. route without producing clinical illness. A short-lived viremia was followed by replication of the virus in the liver, spleen, kidney, and placenta. The virus replicated actively in the placenta and was transmitted to the fetus as shown by isolation of JEV from fetal tissues and newborn infants. These experiments confirmed the transplacental transmission of JEV from mother to fetus in mice and the utility of this model in such studies. The pathogenesis of viral infection in the mouse model closely parallels JEV infection in humans, whereas in swine it usually results in production of symptomless viremia (10). An added advantage is the ease with which mice can be handled.

An extensive outbreak of encephalitis occurred in the human population at Gorakhpur in 1978 (Mathur et al., unpublished data). During this epidemic, we demonstrated the transplacental transmission of JEV in humans by isolating the virus from placenta, brain, and liver tissues of one of the aborted fetuses (2). In a number of viral infections, the placenta is the site of active replication of the virus, with consequent spread to the fetus. Transplacental transmission of some of the arboviruses has been demonstrated in experimental mice: Colorado tick fever (6), bluetongue (9), and Venezuelan equine encephalitis (12) viruses.

Pregnant mice inoculated with JEV during the 1st week of gestation suffered significantly more fetal and neonatal deaths (66%) than did mice inoculated during the 2nd or 3rd week of

TABLE 3. *JEV HI antibody titer before and after treatment with 2-mercaptoethanol in sera of 5-week-old mice*

Group	Total no.	No. of sera showing HI antibody titer of:						No. positive	% Positive
		20	40	80	160	320	640		
A. HI antibody in 5-wk-old mice delivered by mothers that received JEV on day 15 of gestation	9			3	1	1	1	6	66.6
2-Mercaptoethanol-treated sera		3	2	1				6	66.6
B. 5-wk-old control mice	11								

gestation. Minimal damage was seen in offspring of mice infected during the 3rd week of pregnancy. Only 13.8% of the neonates died. Of the healthy offspring of these mothers, 66.6% had HI antibodies, including specific IgM antibodies, in their blood, indicating exposure to JEV in utero. This supports our earlier observation with human females; abortions occurred after infection with JEV early in gestation, whereas women infected near term delivered apparently healthy babies (2). Furthermore, adult pigs are not adversely affected by JEV, but sows infected early in pregnancy deliver stillborn or abnormal young (11). Burns (1) has observed a high incidence of stillbirths in swine associated with the JEV epidemics and epizootics in Japan. It therefore appears that the adverse affect of JEV on the fetus occurs when the infection is acquired early in gestation in mice, humans, and pigs. The adverse effects of Colorado tick fever, Venezuelan equine encephalitis, and bluetongue viruses have been seen more frequently in mice inoculated during the 2nd week of gestation (6, 9, 12) whereas cytomegalovirus effects in pregnant guinea pigs are most pronounced when the virus is contracted late in gestation (5).

The outcome of viral infection during pregnancy depends on three factors: the virulence of the virus, the period of gestation, and the immune status of the mother. In this study, mice were nonimmune. Since virus virulence was the same for all mice, the deciding factor for fetal infection and disease appeared to have been the gestational period. Actively multiplying and immature cells are commonly the target of the virus during congenital infection. It appears likely that JEV affects the immature fetus more severely, and as the fetus matures, it becomes as resistant to peripheral inoculation of the virus as adult mice. Thus, virus replicates but produces no apparent ill effects. Since the virus could be isolated from fetuses, stillborns, and neonates, it appears that fetal death is due to the direct effect of JEV.

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