Intrauterine Infection of Mice with St. Louis Encephalitis Virus: Immunological, Physiological, Neurological, and Behavioral Effects on Progeny

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Intravenous injection of pregnant mice with St. Louis encephalitis (SLE) virus at 8 days of gestation resulted in infection of the fetus. Progeny developed no antibody or tolerance to SLE virus since the viral antigen was cleared by maternal antibody before antibody-forming competence developed in the young. Temporary growth retardation was observed in a number of young at 3 weeks of age. After the initial setback the growth rate increased, indicating that early running was due to an inability to adjust adequately to extrauterine life, which was subsequently overcome. In most other young there were no significant effects on growth, reproduction, or life expectancy. A few young died at or shortly after birth; in these, neurological changes ranging from gross defects such as encephaloceles and hydrocephalus to histological evidence of necrosis and congestion were observed. Neurologically related behavioral changes were detected by using the open field test and the rotating-rodding test, which indicated neurological damage and memory impairment in the surviving intrauterinely infected animals.

Congenital infections due to togaviruses have been reported in both humans and animals (3, 15, 16, 20). Infants born to mothers known to have been infected with Venezuelan encephalitis virus during the 3rd to the 8th month of pregnancy were found to have severe cerebral lesions (20). These were attributed to placental transfer of the virus. The death of twins due to Western encephalitis was attributed to placental transfer: the mother had been heavily exposed to mosquitoes before delivery, and the infants had been in the hospital with no contact with mosquitoes (16).

Previous work with St. Louis encephalitis (SLE) virus in pregnant mice demonstrated the value of this model system for study of placental transfer of arboviruses, since over 80% of the embryos became infected if the mother was injected after day 7 of gestation (1). It is significant that when injection is at 7 to 9 days of gestation many of the young survive to be born and live an apparently normal life. Virus has not been recovered at birth from these young even though it could be detected in high titers in the brain of the embryo shortly after injection of the mother. This extensive prenatal infection with no readily apparent permanent effects led to the investigation of four areas: the mechanism of virus elimination from the fetus, the pathological effects of the transplacental viral infection on brain tissue, the effects of the transplacental viral infection on growth and longevity of the progeny, and the effects of transplacental viral infection on the behavior of the infected progeny, considering a possible analogy with cases of human congenital anomalies believed to be caused by prenatal viral infections.

Studies of immunological, neurological, physiological, and behavioral effects of SLE virus on the progeny were undertaken to determine what effects could be expected after an arbovirus infection during pregnancy. The results imply that epidemiological and neurological studies should be undertaken of children born to mothers who are known to be exposed or infected during epidemics of arboviruses.

(This paper is taken in part from a thesis submitted by A.A.A. to the faculty of the University of Wisconsin in partial fulfillment of the requirements for the Ph.D. degree).

MATERIALS AND METHODS

Virus. Flicker strain (CDC-904; reference 11) of SLE virus has been maintained by intracerebral passage in suckling mice and prepared as previously described (1).

Mice. Swiss white mice of the Charles River Ha/ICR strain were supplied by the University of

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Wisconsin Department of Veterinary Science research farm. Time of breeding was determined by examination for vaginal plugs (1). Experimental mice were from mothers injected intravenously with $10^8$ suckling mouse mean lethal doses of SLE virus on day 8 of gestation, which results in over 90% of the embryos becoming infected by day 12 of gestation (1). Mothers of control animals received similar injections of normal suckling mouse brain.

The experimental procedure for handling the gravid mice followed one of three methods, depending on the objective of the experiment: (i) natural birth with the young raised by their own mothers; (ii) necropy of the mother at day 18 of gestation with the young being raised by nonimmune foster mothers; and (iii) necropy of the mother at various times after injection with samples taken from mother and fetuses for virus and antibody level determination.

**Virus and antibody levels during fetal development.** Each day from day 9 to day 18 of gestation three mice were bled and necropied, and maternal blood and serum were saved for virus and antibody determination. The uterus with the fetuses was removed intact by sterile procedures and repeatedly washed with saline to remove maternal blood. It was then opened; individual fetuses were again washed to remove all surface blood. The fetuses were ground as a 20% suspension and centrifuged. After testing for virus, the remaining supernatant was heat inactivated at 56 C for 1 h and tested for antibody. Control animals were similarly processed.

**Virus titrations.** Fetal tissue and maternal blood were titrated in BHK-21 cells by the colorimetric test (Kuns, Ph.D. dissertation, University of Wisconsin, Madison, 1962). End points were calculated by the method of Reed and Muench (12). Isolates were identified by neutralization with known rabbit SLE antiserum.

**Antibody titrations.** Samples were heat inactivated at 56 C for 1 h, after which antibody titers were determined by the colorimetric test, using twofold dilutions.

**Challenge studies.** Mice surgically removed from their mothers on day 18 of gestation were challenged at 4, 14, 28, or 35 days of age by intraperitoneal injection. Challenge studies were also performed at 60 days and 8 months in a group raised by their mothers. The difference in mean lethal dose for SLE virus in the control and experimental animals was used as the measure of resistance at 4 and 14 days of age. Percentages dying instead of end points were calculated for the challenges after 28 days of age, since mice are partially resistant by then. Four intrauterinely infected and three control mice were tested for the presence of antibody at 8 months of age.

**Growth rates.** Mice in growth rate experiments were surgically removed from their mothers on day 18 of gestation and placed with nonimmune nursing mothers. At 3 days of age, 60 experimental and 60 control young were randomly selected, assigned numbers by drawing, and randomly placed 10 to a foster mother. This procedure served to eliminate maternal effects and to eliminate experimenter bias in weighing. The young were weaned and sexed at 21 days of age. Individual weights were recorded at 21, 42, 60, and 100 days.

A second group of 120 mice, raised primarily to be given the horizontal rotating-rod test, was randomized identically. These mice were weighed at 21 days of age, and these weights were analyzed with those of the above group. Male and female weights were not separated since the difference at 21 days is slight and since the numbers of each sex were approximately equal.

**Reproductive ability.** Control young and young of mothers receiving SLE virus at 8 days of gestation were raised naturally until 3 months of age and then placed two females with one male for breeding. At parturition the number of days that had elapsed after initial placement with a male and the number of young found alive were recorded. One group of 24 experimental and 22 control mothers was allowed to raise all its young to 1 week of age. The young of 20 experimental and 19 control mice were redistributed after birth so that each female raised eight young. At 21 days of age the young were individually weighed. In the analysis, weights of young were used only from mothers that raised eight young to 21 days.

**Behavioral testing.** The young given behavioral tests were raised by two methods. In the first method, minimally handled young to be given the open field test were raised by their mothers until 21 days, when the mice were weaned, sexed, and transferred to group cages. In the second method, repeatedly handled young to be used in a second open field test and in all of the rotating-rod experiments were raised on foster mothers. The pregnant natural mothers were necropied on day 18 of gestation and the young were transferred to foster mothers. At 3 days the young were randomly numbered and randomly redistributed 10 to each foster mother. This method had the advantage that both control and experimental animals had an equal chance of being raised by a particular mother, eliminating both handling and maternal nursing effects.

**Open field test.** The open field apparatus was designed and operated similarly to the report of Broadhurst (2). Animals were individually tested in random order for 2 min per day for 5 consecutive days. The number of blocks entered (ambulation score) and the number of fecal pellets excreted were recorded. Average numbers of each measure were calculated for each mouse for runs 2 through 5. Data from run 1 were discarded, since each mouse's response to the initial experience in the apparatus was highly individual and inconsistent with the mouse's later relatively consistent performances in runs 2 through 5. The data were analyzed by a three-way factorial analysis of variance (17).

**Rotating-rod test.** A horizontal rotating rod constructed similarly to that used by Seamer and Peto (14) was used as a test of coordination. At the 27th day of age 200 mice, 50 males and 50 females each of both experimental and control animals, were randomly selected and tested for 5 consecutive days. They were placed on a stationary rod; then the rod was rotated for 3 min at 15 rpm. Mice were replaced
as rapidly as they fell, and the number of falls for each mouse recorded. The sum of trials 2 through 5 was used in the analysis.

Histological examination. A number of young that died in the first hours after surgical birth were fixed in 10% formalin for histological examination. Transverse and longitudinal sections of the brain were stained with hematoxylin and eosin.

RESULTS

Fetal and maternal virus and antibody levels. Virus and antibody levels in the fetuses from mothers injected on day 8 of gestation were determined by the colorimetric test (Fig. 1). Virus first appeared in the fetus around day 11 or 12 of gestation, 3 to 4 days after injection of the mother. Titters rapidly rose to more than 10^2 mean tissue culture doses per g of tissue and remained high from day 13 to day 16. Antibody was first detected in the fetus on day 17 or 18, and titers rapidly rose to a level of 1:80 by day 18. Virus was not detected in any sample after the demonstration of antibody.

No virus was detected by the colorimetric test in any of the 30 maternal blood samples taken at time of necropsy. Maternal antibody was first detected in the serum at day 15 of gestation, 7 days after injection, in low titers (1:5 and 1:20 dilutions) in two animals. The nine mothers that were necropsied from day 16 to day 18 all had antibody titers of 1:10 to 1:80.

Challenge of mice surviving in utero infection. The level and duration of resistance to SLE virus was determined by challenge in mice from mothers injected on day 8 of gestation. To prevent the transfer of antibody through the milk, young were surgically removed from their mothers and transferred to nonimmune nursing mothers. Simultaneous titrations of SLE virus in control and experimental young at 4 days of age demonstrated that young from SLE virus-injected mothers withstood over 10^4.6 suckling mouse mean lethal doses. Mice challenged at 14 days withstood 10^3.6 suckling mouse mean lethal doses of virus. At 28 days, 5 of 15 (33%) of the experimental mice died and 8 of 15 (53%) of the control mice died. Challenges at 35 days resulted in 6 of 21 (29%) of the experimental animals dying, compared with 7 of 21 (33%) of the control animals.

At 60 days of age, 15 each of experimental and control male progeny who had been nursed by their own mothers were challenged with SLE virus. No deaths occurred in the intravenously infected group; five occurred in the control group. At 8 months no neutralizing antibody was detected in five male progeny from injected mothers or in three controls. The remaining nine experimental and nine control mice were then challenged with SLE virus. Five of the experimental group and six of the control group died. Surviving experimental and control animals had neutralizing antibody at levels of 1:40 to 1:320 when tested 3 weeks after challenge.

Growth rates. Growth rates of experimental male and female animals were compared with controls at 21, 42, 60, and 100 days of age (Fig. 2). Experimental male mice consistently weighed less than the male controls at each weighing, but because of variance in weights within groups and the small number of animals, the difference was not statistically significant. Female weights were similar for the experimental and control groups.

The average 21-day weights for males and females were similar (males, 10.5 g; females, 10.2 g), and there were approximately equal numbers of each sex; thus male and female

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**Fig. 1.** Virus and antibody levels in fetal tissue after intravenous injection of the mother on day 8 of gestation. Symbols: ▲, Mean virus titer of fetuses of the three mothers necropsied on each day; ●, individual virus titer of fetuses of each mother; ■, mean antibody titer of fetuses of the three mothers necropsied on each day.
weights were combined in Fig. 3 and for a weight distribution analysis by the Pearson $\chi^2$ test. At this age 29 experimental animals and only 8 control animals weighed 8 g or less (Fig. 3), producing a highly significant shift in the weight distribution curve (Pearson $\chi^2 = 61.27$, significant at $P = 0.01$; reference 5).

Reproductive ability. Forty-eight experimental and 47 control females were mated with experimental and control males, respectively. The mean number of days elapsed between placement with the males and parturition was 22.1 days for 46 experimental mothers and 21.3 days for 44 controls ($t = 1.289$, not significant at the 0.05 level).

When litter sizes were compared, mean numbers of young found alive after birth were 10.8 per litter for the 46 experimental females and 12.1 per litter for the 44 controls ($t = 1.368$, not significant at the $P = 0.05$ level). The median was 12 for both groups.

At birth 24 experimental mothers and 22 control mothers were allowed to raise all their young for 1 week to determine the number surviving compared with the number born. The experimental mothers had a mean of 12.5 young and raised 11.8, a mean death rate of 0.7 young per litter. Controls had 13.5 young and raised 12.5, a mean death rate of 1.0 per litter for the first week, essentially the same as the experimental data.

With the remainder of the females the young were redistributed at 1 day of age to obtain a uniform eight young per litter. Seven experimental and nine control mothers raised the eight second-generation young for the total of 21 days. The mean weaning weight of the young was 10.2 g for the experimental mothers and 10.6 g for the controls, results that show no appreciable difference.

In the group of 24 experimental and 21 control young that were held until 8 months of age, one control male died at 7 months and no deaths occurred in the experimental group.

Pathology of the brain. Microscopic examination of tissue sections from experimental young that died shortly after surgical birth and placement with the nursing foster mother were compared with sections from control mice of the same age. Direct comparison was found to be necessary because of the large number of proliferating basophilic cells seen in the brain at birth. Figure 4 is of normal cerebral brain tissue in the 18-day fetus, which is neuron rich and exhibits normal architecture.

Experimental animal no. 85 was a fully developed live infant mouse that was surgically removed from the mother on day 18 of gestation and died within a few minutes of birth. Figure 5 is a general view with softening of the brain near the lateral ventricles, in which necrosis of the choroid plexus and ependymal is obvious. Figure 6 is an enlarged view of part of Fig. 5, at the edge of the softening. Marked karyorrhexis and pyknosis are evident in the section.

Experimental animal no. 92 (Fig. 7) died 2 h after surgical removal on day 18. The figure is of a focus of necrosis in the cerebrum characterized by pyknosis and karyorrhexis with marked hyperemia in the capillaries. The necrotic cells are either spongiosiolasts or glioblasts. The ependyma at the left is relatively intact.

Experimental animal no. 88 (Fig. 8) was also surgically removed on day 18. It had an encephalocoele but was alive at birth. Figure 8 is a section of the cortex with a number of dead...
FIG. 4. Section of cerebrum of a normal fetus on day 18 of gestation. Hematoxylin and eosin stain. ×800.

FIG. 5. Section of cerebrum of experimental SLE fetus on day 18 of gestation, with softening (×) of brain near lateral ventricles (○) and necrosis of ependyma (→). Mother was injected with SLE virus on day 8 of gestation. Hematoxylin and eosin stain. ×128.
**Fig. 6.** Enlargement from Fig. 2, section of cerebrum of experimental SLE fetus at edge of softening (×), with marked karyorrhexis and pyknosis evident. Hematoxylin and eosin stain. ×800.

**Fig. 7.** Section of cerebrum of experimental SLE fetus with necrosis characterized by pyknosis and karyorrhexis and by marked hyperemia in the capillaries. Hematoxylin and eosin stain. ×800.
FIG. 8. Section of cerebrum of experimental SLE fetus with dead neurons (→). A few relatively unaffected neurons are indicated near the top (×). Hematoxylin and eosin stain. ×800.

neurons near the center. In the top center are a number of neurons that are relatively unaffected.

**Gross defects.** Grossly visible defects were observed in a few young from mothers that received SLE virus during gestation. These defects were limited to neuroanatomic changes and consisted of encephaloceles and hydrocephalus. Encephaloceles (Fig. 9) were observed in nine young from over 300 mothers that received virus from the 4th to the 9th day of gestation. Two of these young, observed at necropsy of the mother on the 16th day of gestation after injection on day 8, had virus titers of over $10^{5.5}$ mean mouse infective doses. The seven other encephaloceleic mice were observed at the 18th day. Three of these were tested for virus with negative results. Histological sections taken from one other mouse contained isolated necrotic cells in widely separated areas of the brain and microscopic hemorrhage around the edge of the encephalocele. No encephaloceles were observed in any young from an approximately equal number of control mothers.

Hydrocephalus was observed in eight young, all of which had gross evidence of hydrocephalus on preliminary examination. The evidence was confirmed by necropsy or by histological examination of the brain. Figure 10 is of a group of infant mice from a litter with dead and live fetuses at various stages of development, including a fetus that has hydrocephalus. At necropsy this fetus was found to have enlargement of the lateral ventricles. Histological examination of other young has also shown enlargement of the lateral ventricles or of the third ventricle.

**Behavioral testing: open field test.** Results (Table 1) are the means of the average daily ambulation scores of each group of 24 animals. Group 1 mice received minimal handling and group 2 mice had been handled repeatedly. The
data were analyzed by factorial analysis (17), comparing group 1 against group 2, male against female, and experimental against control. The means of group 1 and group 2 were significantly different ($F = 114.6$), and experimental and control means were significantly different ($F = 17.38$); the difference between males and females was insignificant ($F = 0.06$). Upon analysis of the interactions, the experimental versus control significant difference was found to be due to group 2 rather than group 1.

Fecal pellet counts were recorded at the same time as the ambulation scores. These counts were not significantly different, either between handling groups, between experimental animals and controls, or between males and females.

Rotating-rod test. The total number of falls for trials 2 through 5 were added to give a score for each individual mouse. The mean number of falls for 50 experimental males was 10.3 falls; for 50 controls, it was 5.9 falls. The mean number of falls for 50 experimental females was 9.5 falls; for 50 controls, it was 7.9 falls. Individual scores were grouped into frequency distributions for males and females (Fig. 11). Comparison of the experimental and control male distributions by the Pearson $x^2$ gave a $x^2$ of 20.61, significant at $P = 0.01$. The comparison of the female distributions was not significant ($x^2 = 6.53$).

**DISCUSSION**

Previous work (1) with SLE virus in pregnant mice had demonstrated that the virus would cross the placenta and infect the fetus, with the outcome of the infection dependent on the time of injection of the mother. Injection after 10 days of gestation usually resulted in death of the fetus at the time of birth or shortly after, with virus being readily recovered from the fetus. Before day 9 of gestation the fetuses became infected but eliminated the virus before birth, and many of the live-born progeny led an apparently normal life.

Our studies with placental transfer of SLE virus indicate that antibody is transferred in utero, since the clearing of virus from the fetus follows the detection of antibody in the mother and subsequent rise in antibody level in the fetus.

Further evidence for placental transfer of maternal antibody is that infant mice surgically removed from their own mothers and raised by nonimmune foster mothers were resistant to SLE virus when tested at 14 days. This resistance to SLE virus diminished until 35 days of age, when the experimental mice were again susceptible to challenge.

Transplacental infection provides an excel-

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**TABLE 1. Blocks entered per 2-min run in open field test**

<table>
<thead>
<tr>
<th>Determinationa</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SLE</td>
<td>Control</td>
</tr>
<tr>
<td>Group 1</td>
<td>14.2</td>
<td>17.3</td>
</tr>
<tr>
<td>Mean</td>
<td>14.5</td>
<td>17.8</td>
</tr>
<tr>
<td>Median</td>
<td>24.6</td>
<td>34.8</td>
</tr>
<tr>
<td>Group 2</td>
<td>21.6</td>
<td>38.6</td>
</tr>
</tbody>
</table>

*a Data are means from four runs on consecutive days, 24 animals in each group. Mothers of SLE virus were injected intravenously with $10^6$ suckling mouse mean lethal doses of SLE virus on day 8 of gestation.

*b Group 1 mice were minimally handled; group 2 mice were repeatedly handled.

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**FIG. 10. Young removed from one mother on day 18 of gestation. Left-hand fetus in lower row has hydrocephalus, which was confirmed by necropsy. Mother was injected with SLE virus on day 8 of gestation.**
lent opportunity for the establishment of a state of tolerance or a persistent infection in the mouse. The virus was present during much of the embryonic developmental period, and pathological studies on mice dying at birth showed a lack of cellular response even though widespread necrosis was seen. There was no indication that either tolerance or a persistent infection followed the exposure to SLE virus, since the response to challenge of progeny infected in utero and nursed by nonimmune mothers was the same as that of control animals at 35 days of age. In addition, no neutralizing antibody was detected at 8 months in intrauterinely infected mice raised by their mothers. Challenge of experimental and control mice at 8 months gave similar death patterns, and mice which survived had a normal antibody response to the challenge.

The finding of an absence of active antibody production after fetal infections without immune tolerance has been described by St. Geme et al. (13) with mumps virus in monkeys and by Kilham and Margolis (9) with reovirus type 3 in rats. Kilham and Margolis hypothesize that the fetal infection with reovirus type 3 was largely limited to fetal skin and mucosa, failing to reach deeper-lying sites of immunocyte proliferation where either antibody formation or an immune tolerance might be induced. This explanation is not appropriate to fetal infection with SLE virus, since a direct infection of the em-
other young with encephaloceles were discovered on day 18 of gestation, when virus is no longer routinely found in the fetus (1). Histological sections of the brain of one encephalocele fetus had isolated necrotic cells similar to those seen in young that die shortly after birth.

Hydrocephalus, the other gross defect seen, is exceptionally hard to evaluate as a teratological effect of SLE virus infection, since a ventricular dilatation must be severe before it can be observed. Thus many young could have mild degrees of hydrocephalus and not be detected. The short gestation of the mouse, which may not permit full development of hydrocephalus before birth, may also contribute to the low incidence.

The open field test, designed to measure emotional reactivity and exploratory drive (21), was used to evaluate less evident neurological changes. The results of the first run with mice that had received minimal handling indicated that there was a trend toward lower ambulation scores in the experimental mice. This was seen in both the males and the females; the lower scores would indicate higher emotionality in the experimental animals than in the controls (21). A second group, handled repeatedly for weighing and rotating-rod testing, had consistently higher ambulation scores than the first group. This was expected, since infant handling is known to produce significantly higher ambulation scores (4, 18), although the reason is not fully understood (18). Control of fear is thought to be a large factor, since free plasma corticosterone levels after testing are inversely proportional to the ambulation score (10).

The unexpected finding in our study was that the differences in ambulation scores between the infected and noninfected mice were increased by handling. This suggests that the experimental animals were not able to respond, or "learn," as well as the controls from the conditioning by handling. If fear is a factor in the test, it is reasonable to assume that memory of the outcome of previous handling should reduce the fear on subsequent tests and result in higher scores. The finding of lower ambulation scores in the experimental mice would then imply an impaired memory in the experimental mice.

The second behavioral test, the rotating-rod test, evaluated the locomotor and balancing ability of the mouse, based on mean scores of repeated runs (14, 19). Learning is considered a factor, since there is improvement in the ability of a mouse to remain on the rod between the first and the succeeding runs (14). The analysis showed that there was a significant ($P = 0.01$) shift toward more falls per mouse in the experimental males; females showed the same trend, with the difference approaching significance at the $P = 0.05$ level.

The trend toward a high number of falls in the experimental mice indicates either a neurological or a physical difference from the controls. Muscular deficiencies could result in more falls because the physically impaired mouse would not be as able to keep up with the turning rod. However, growth rates, life expectancy, reproductive ability, and general behavior gave no evidence of such a defect. The possibility of neurological deficiency is more likely since both the open field test and histological studies indicate neurological damage.

The results of the histological examination of brain tissue and the observation of gross neurological defects, along with the finding of behavioral changes and high virus titer in the brain, demonstrate that SLE virus is a neurotropic virus in the fetal mouse. Any degree of involvement from death to no readily observable permanent effect can be expected in individual progeny after intrauterine infection with the virus. All teratological studies with neurotropic viruses must include assays for virus and careful examination of the fetus at various times after infection, supplemented by neurological and behavioral studies of the surviving animals to evaluate lesser degrees of damage. It is also imperative that these studies include large numbers of animals, since the teratological effects may be demonstrable in only a limited number of the experimental animals.

The brain has a large reserve capacity and can compensate for neurological damage in many individuals, but this study has indicated that with SLE virus infection damage can be severe enough to produce demonstrable neurological deficiency when death does not occur. The behavioral changes seen could be analogous to those that occur in cases of human minimal brain damage. This is particularly evident when examining performances that are felt to be dependent upon memory. Epidemiological and neurological monitoring of children exposed prenatally to arbovirus epidemics should be undertaken, with follow-ups conducted at intervals including the early elementary school years because of the possible effect of minimal brain damage upon the child's ability to attend or upon his ability to comprehend and produce either spoken or written language. The existence of the learning-disabled child is now recognized by educators; prenatal togavirus infections should be recognized and investigated as a possible cause of learning disability.
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LITERATURE CITED


