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Effect of Infection with the *ts*22 Mutant of Semliki Forest Virus on Development of the Central Nervous System in the Fetal Mouse

MOHAMED J. E. M. F. MABRUK,[1] GWENDOLINE M. GLASGOW,[1] ALMA M. FLACK,[2] JEAN C. FOLAN,[3] JOHN G. BANNIGAN,[3] JOAN M. B. SMYTH,[1] M. AIDEEN O'SULLIVAN,[1] BRIAN J. SHEAHAN,[2] AND GREGORY J. ATKINS[1]*

Department of Microbiology, Moyne Institute, Trinity College, Dublin 2,[1] Department of Veterinary Pathology, Faculty of Veterinary Medicine, University College, Dublin 4,[2] and Department of Anatomy, Faculty of Medicine, University College, Earlsfort Terrace, Dublin 2,[3] Ireland

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The A7 strain of Semliki Forest virus induces rapid fetal death in pregnant mice, whereas the *ts*22 mutant derived from it is teratogenic for a proportion of fetuses. Both A7 and *ts*22 induce viremia and infect the central nervous systems and fetuses of pregnant mice. Using immunogold-silver staining, a cDNA probe for a Semliki Forest virus nonstructural sequence, and a riboprobe derived from the same sequence, we showed that the skin and musculoskeletal systems of fetuses from mothers infected with *ts*22 were often heavily infected but the central nervous systems were not labeled before day 17 of pregnancy. Damage to the neural tube, including open-neural-tube defects, was detected in fetuses following infection of the mother at days 8 and 10 of pregnancy with both A7 and *ts*22. For *ts*22, neural tube damage induced by fetal infection before day 17 of pregnancy appeared to be indirect and caused by virus infection of mesenchymal cells surrounding the developing neural tube.

Semliki Forest virus (SFV) infection of laboratory mice has been extensively studied as a model of viral pathogenicity (3). Virulent strains, such as L10, cause lethal encephalitis in weanling mice, whereas avirulent strains, such as A7 (an independent isolate of SFV) and the M9 mutant (derived from L10), allow survival of most infected mice but induce central nervous system (CNS) demyelination (3, 6).

Although the A7 strain of SFV is avirulent for weanling or adult mice, it is lethal for developing fetuses following peripheral infection of the mother (1). The mode of fetal infection is induction of viremia followed by infection of the placenta (9, 17). We have used A7 as a model to analyze embryonic and fetal infections (8, 9). We isolated four mutants of A7 which differed from the wild type in their effects on developing mouse fetuses following infection of the mother. One mutant, *ts*22, was teratogenic in a proportion of fetuses rather than rapidly lethal like the wild type. Compared with that of the wild-type A7 strain, multiplication of *ts*22 is restricted at 39°C but not at 37°C, and this temperature sensitivity results from a defect in viral RNA synthesis (9). We have shown that a large proportion of skeletal and skin defects are induced in mouse fetuses following infection of the mother at days 8 and 10 of pregnancy with *ts*22. Using immunogold-silver staining (IGSS) and in situ hybridization with a cDNA probe, we showed that this correlated with a tropism of *ts*22 for mesenchymal cells of the developing dermis and surrounding cartilaginous plate (15). One surprising finding was that the CNS was poorly stained with these probes, since the wild-type A7 strain is neurotropic for adult mice (6, 19). We further investigated infection of mouse fetuses by *ts*22 and showed that infection of a fetal CNS cannot be detected before day 17 of pregnancy by using antibody, DNA, or RNA probes, but infection of a fetus with *ts*22 can lead indirectly to induction of neural tube defects.

* Corresponding author.

MATERIALS AND METHODS

Virus. A plaque-purified stock of the *ts*22 mutant of SFV was grown in BHK-21 cells at 30°C as previously described (2). This stock virus was plaque assayed and frozen in aliquots at −70°C. A similar stock of the wild-type A7 strain was grown at 37°C.

Mice. The randomly bred Q/Fa mouse strain was used, since this produces large numbers of fetuses per mother. To produce pregnant mice of known gestation, males and females were placed together in a ratio of 2:1. The day when a vaginal plug was found was taken to be day 1 of pregnancy.

Infection experiments. Pregnant mice were injected intraperitoneally with 10^4 PFU of virus in 0.5 ml of phosphate-buffered saline. The virus contents of fetuses and brains were measured with 10% (wt/vol) clarified tissue homogenate and with blood by using a 10% (vol/vol) suspension as previously described (5).

Fetal pathologic changes. For histopathologic examination, IGSS, and in situ hybridization, fetuses from triplicate mothers infected on day 10 of pregnancy with *ts*22 were examined at daily intervals from days 12 to 17 of pregnancy. The fetuses were fixed as described by Mabruk et al. (15) and embedded in paraffin wax. Parallel sections were cut in a sagittal plane at 4 μm and examined by light microscopy. Sections for histopathologic examination were stained with hematoxylin and eosin; sections for IGSS and in situ hybridization were lightly counterstained with 2% methyl green. To investigate the effect of infection with *ts*22 or A7 on neural tube closure, mothers were infected at day 8 of pregnancy, since the neural tube closes at day 10 during normal mouse fetus development (21). For *ts*22, at least three fetuses from each of three infected mothers were taken at days 11, 14, and 17 of pregnancy. For A7, three fetuses were taken from three infected mothers at day 11 only, since necrosis of all fetuses occurs at subsequent times. As controls, three fetuses were taken from each of two uninfected mothers at days 11, 14, and 17 of pregnancy. The fetuses were dissected free of their membranes and fixed overnight at 4°C in 2% paraformalde-

hyde–2% glutaraldehyde–0.1 M phosphate buffer. Specimens were postfixed for 1 h in 1% osmium tetroxide, dehydrated in ascending concentrations of ethanol, and embedded in Epon. Semithin (1 μm) transverse sections (160 for each fetus) were taken from the lumbar region and stained with toluidine blue. Ultrathin sections were contrasted with uranyl acetate and lead citrate (22) and observed with a Philips 201 electron microscope.

IGSS. IGSS was performed by using purified rabbit anti-SFV immunoglobulin G, gold-labeled anti-rabbit immunoglobulin G, and a silver intensification kit (Janssen Life Sciences Ltd., Olen, Belgium) as described by Mabruk et al. (15).

In situ hybridization. The procedure for in situ hybridization with a cDNA probe for SFV nonstructural sequences has been described by Mabruk et al. (15). A negative-stranded RNA probe (riboprobe) for the same SFV nonstructural sequences was produced by subcloning of the cDNA sequence into a Gemini vector. The 484- and 488-base-pair fragments from a *Pst*I digest of plasmid pKTH301 (corresponding to sequences from SFV 18S defective interfering RNA [14]) were subcloned into the *Pst*I site of the Gemini II vector (Promega Biotec, Madison, Wis.), and the orientation was determined by sequence analysis. The plasmid was linearized by digestion with *Acc*I, and a negative-strand RNA transcript of 87 bases labeled with [^{35}S]UTP was prepared. After digestion with DNase, the digested plasmid and unincorporated nucleotides were removed by centrifugation through a Sephadex G50 (Pharmacia) column (16). The riboprobe was applied to sections at 10^4 cpm/μl (approximately 100 pg of RNA per μl).

RESULTS

Multiplication of A7 and *ts*22 in pregnant mice. To compare multiplications of A7 and *ts*22 in pregnant mice and to test the neurotropism of *ts*22 and A7 in pregnant adult mice, groups of mice were infected with both virus strains at day 10 of pregnancy. Fetal, brain, and blood samples pooled from three mice were taken at daily intervals from days 12 to 17 of pregnancy and assayed. The results are shown in Fig. 1. This indicates that *ts*22, like A7, does multiply in the brains as well as the fetuses of pregnant mice.

Histopathologic changes. No significant lesions were seen in sagittal sections of fetuses from control and *ts*22-infected mothers examined on days 12, 13, 14, 15, and 16 of pregnancy.

Of 14 17-day-old fetuses from infected mothers, 12 showed widespread necrosis of the skin, striated muscle, and adjoining cartilaginous plates. The skin lesions were characterized by necrosis and edema in the dermis, with occasional pycnotic nuclei in the epidermis and vacuolar degeneration of keratinocytes (Fig. 2a). The epidermis in these fetuses was two to three cells thick and showed poorly developed dermal invaginations, in contrast to the six to seven layers of epidermal cells and well-developed dermal invaginations in the skin of 17-day-old control fetuses. Myocardial and skeletal muscle fibers were swollen, and many showed prominent nuclear pycnosis or karyorrhexis (Fig. 2b). Pycnotic and karyorrhectic nuclear debris was also prominent in the mesenchyme immediately surrounding cartilaginous plates (Fig. 2c). Ossification was less advanced in these cartilaginous plates than in those of control fetuses. No evidence of necrosis was seen in brains and spinal cords.

IGSS and in situ hybridization. The results of IGSS and in situ hybridization (for selected fetuses) using the cDNA probe for fetuses from mothers infected on day 10 of pregnancy have been previously described by Mabruk et al. (15). We examined all 94 fetuses in the latter study by using the cDNA probe. Sixty-three fetuses taken at the same times from mothers injected with phosphate-buffered saline only were also examined; none of these showed positive staining. The percentage of positive fetuses by IGSS and in situ hybridization with the cDNA probe is shown in Fig. 3. Although IGSS-positive fetuses were detected mostly on day 17 of pregnancy, a proportion of fetuses was positive by the cDNA probe at every time point. No fetuses were positive by IGSS and negative by in situ hybridization. Fetuses positive by both IGSS and cDNA hybridization showed similar patterns of staining.

Five fetuses which were positive and 13 which were negative by cDNA hybridization were examined by using the riboprobe. All fetuses which were positive by cDNA hybridization were also positive by the riboprobe. No fetuses were detected which were positive with the riboprobe and negative with cDNA or vice versa. Five fetuses from mothers injected with phosphate-buffered saline only were negative with the riboprobe.

For positive fetuses, the most intense concentrations of viral antigen and nucleic acid occurred in the skin, around cartilaginous plates, and in muscle (Fig. 2d). The CNS was unstained before day 17, although mesenchymal cells adjacent to the neuroepithelium did show staining (Fig. 4). For

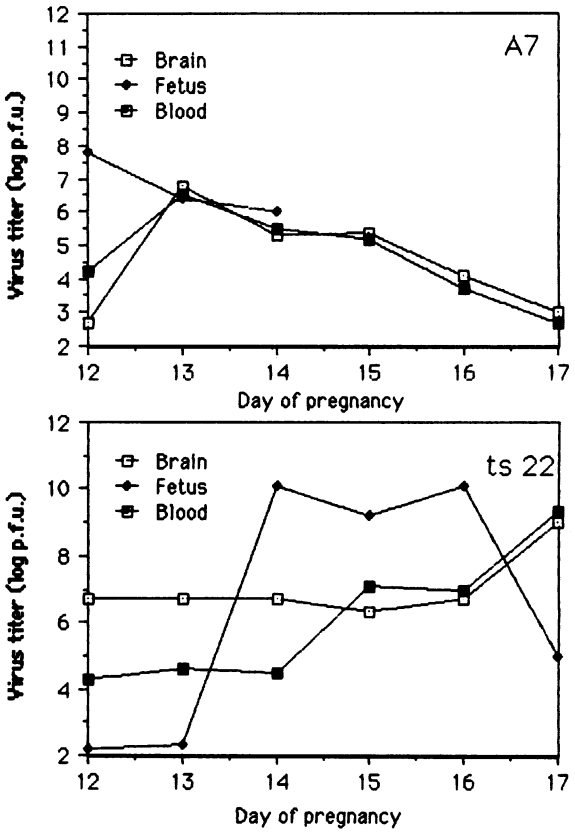

FIG. 1. Multiplication of A7 and *ts*22 in pregnant mice. Mice were infected intraperitoneally at day 10 of pregnancy with 10^4 PFU; virus titers are given as PFU per gram for brains and fetuses and as PFU per milliliter for blood. Fetal death occurred subsequent to day 14 for A7.

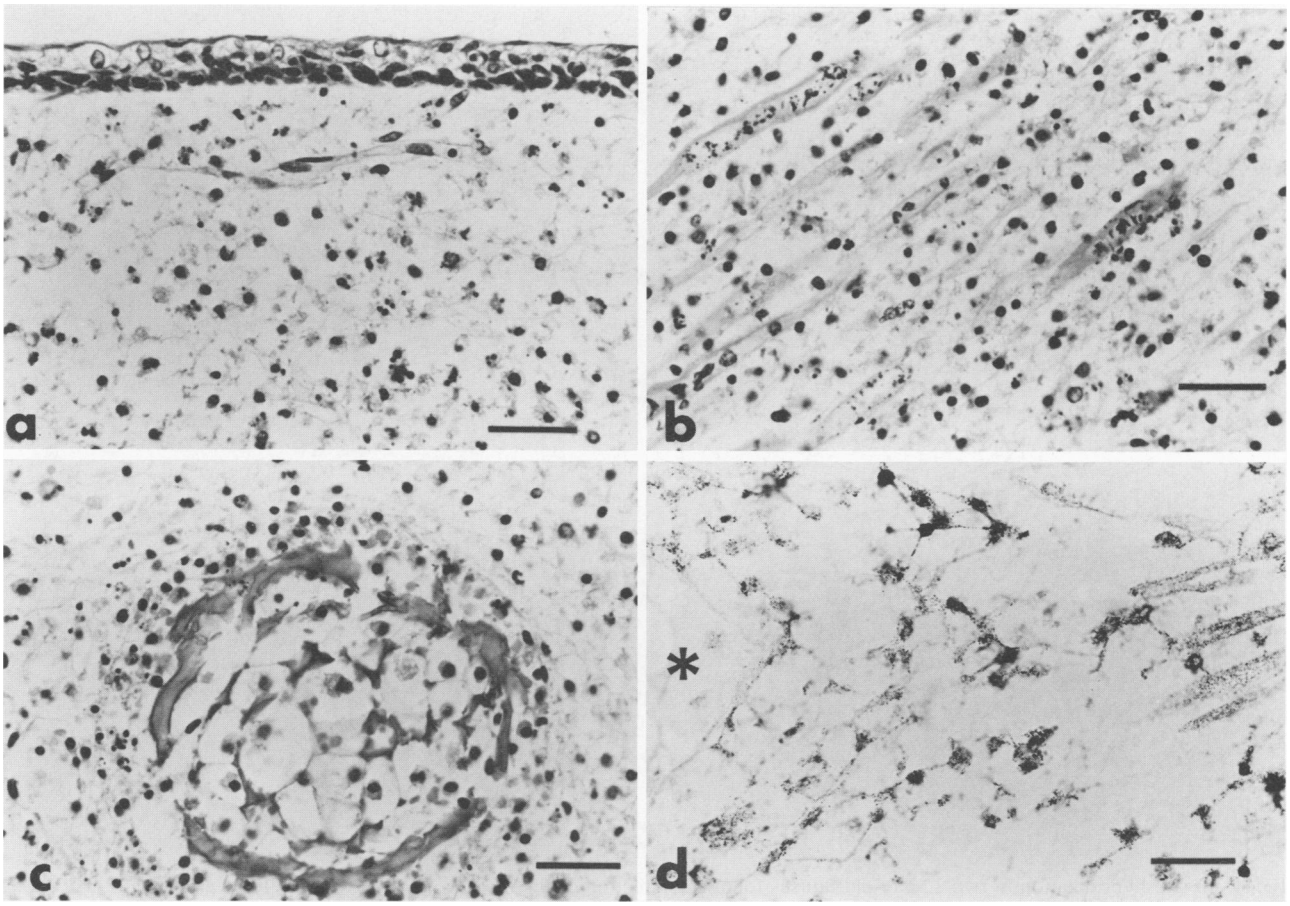

FIG. 2. Tissues from 17-day-old fetuses following infection of the mother with *ts*22 on day 10 of pregnancy. (a) Necrosis and edema in the dermis with vacuolar degeneration of epidermal keratinocytes stained with hematoxylin and eosin; (b) necrosis and swelling of skeletal muscle fibers stained with hematoxylin and eosin; (c) necrotic cell debris around cartilaginous plate stained with hematoxylin and eosin; (d) stellate mesenchymal cells and elongate muscle fibers adjoining a cartilaginous plate (*) immunostained with rabbit polyclonal anti-SFV immunoglobulin G. Panels a, b, and c show tissues from a single fetus, and panel d is from a separate fetus; both fetuses showed undamaged CNS tissue. Bars, 4 μm.

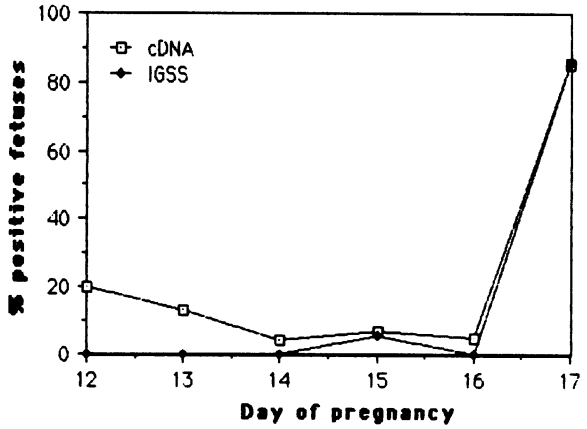

FIG. 3. Comparison of the sensitivity of in situ hybridization with that of a cDNA probe and antibody labeling by IGSS for fetuses from mothers infected with *ts*22 on day 10 of pregnancy. Mothers were killed and fetuses were removed on the day of pregnancy indicated; the data for IGSS are from Mabruk et al. (15).

day 17 fetuses, some staining of the CNS did occur, but this was generally less intense than staining of other tissues. The distribution of viral antigen and nucleic acid in fetuses from infected mothers correlated with areas of necrosis.

Although sagittal sections were used in this study to show the general distribution of viral nucleic acid and antigen, neural tube defects are more easily detected by transverse sections (see below). However, we were able to detect ventral neural tube defects in two fetuses sectioned sagittally at day 12 of pregnancy following infection of the mother at

TABLE 1. Screening of fetuses from infected mothers for viral antigen[a]

Day of pregnancy infected[b]	Day fetuses examined	No. positive/ total examined
8	11	0/15
	14	0/25
	17	25/25
12	15	0/21
	17	8/22

[a] Fetuses were taken from three infected mothers for each time point.
[b] The data for day 10 are from Mabruk et al. (15).

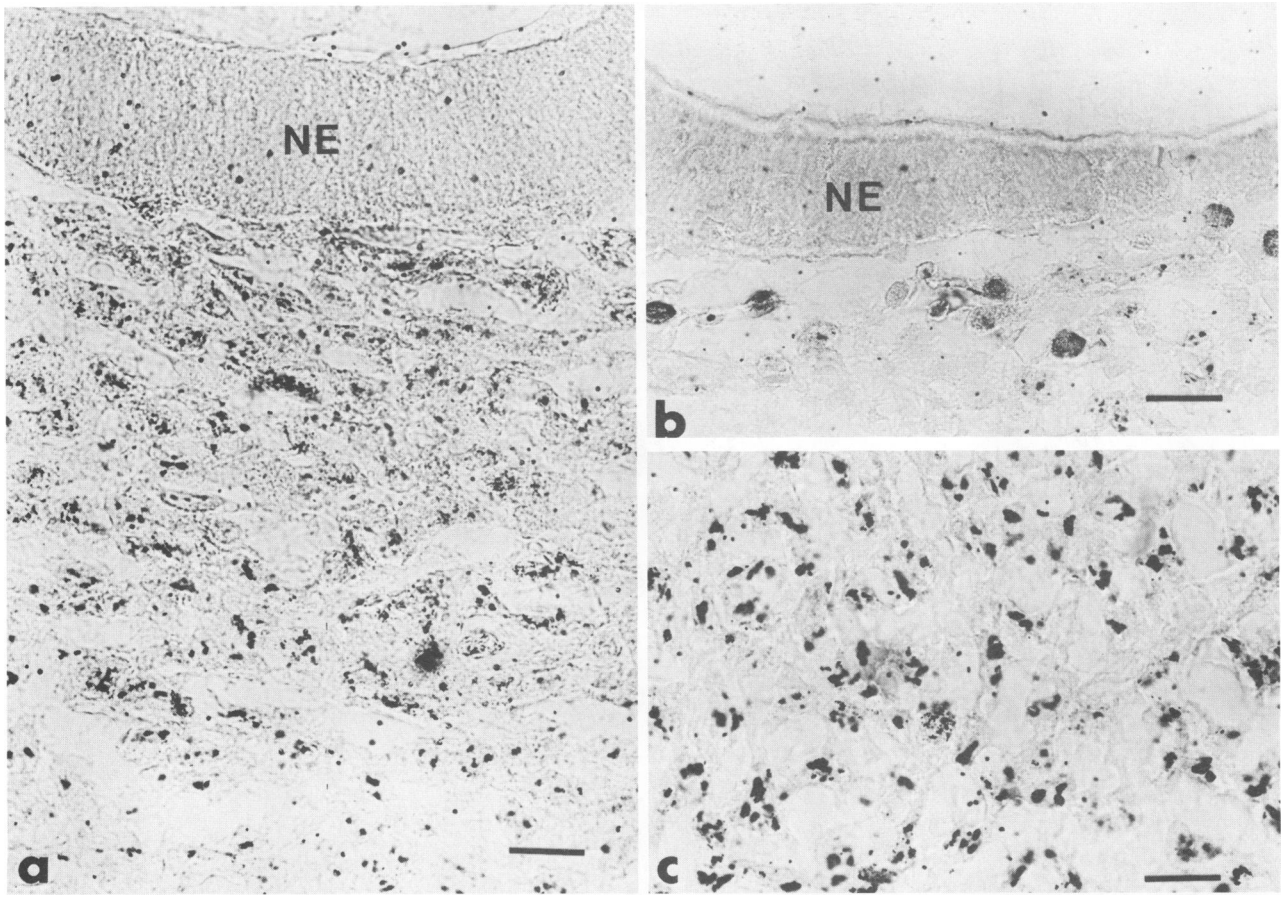

FIG. 4. In situ hybridization in two 12-day-old fetuses following infection of the mother with *ts*22 on day 10 of pregnancy. (a) Fetus 1. Mesenchymal cells adjoining the neuroepithelium (NE) are labeled with [^{35}S]cDNA to SFV nonstructural sequences. (b and c) Fetus 2. Relative sparing of the neuroepithelium (NE; b) and positive labeling of mesenchymal cells in the dermis (c). Bars: a, 10 μm; b, 4 μm; c, 10 μm.

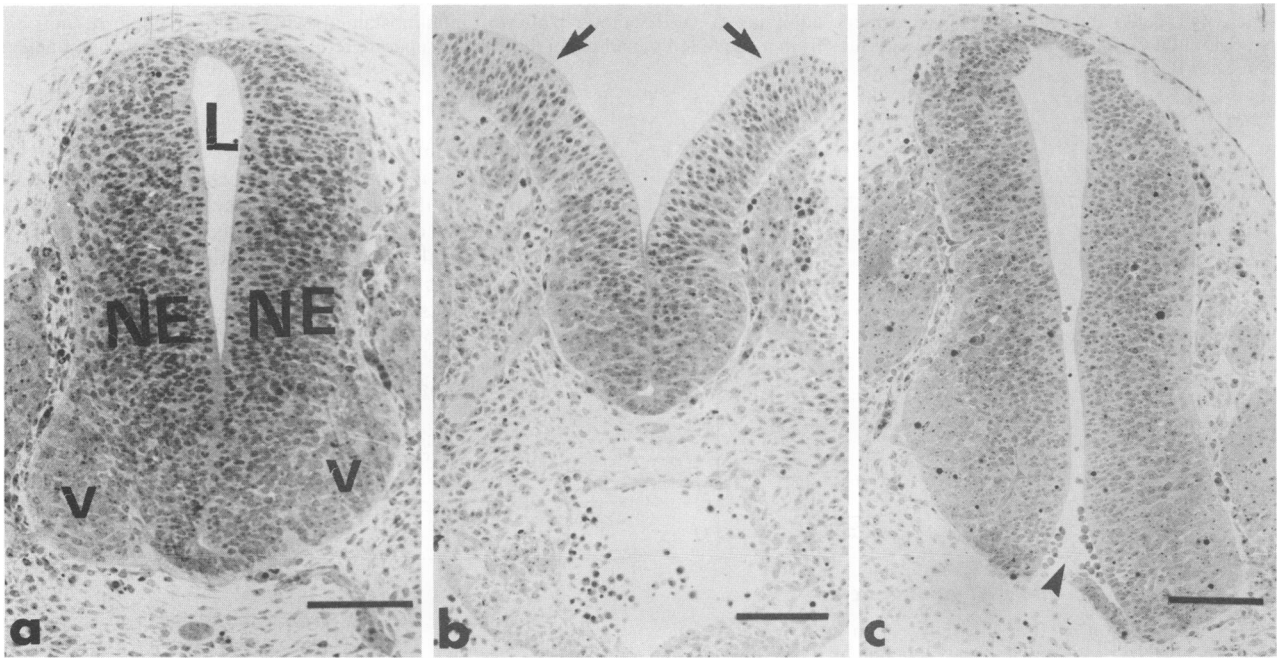

FIG. 5. Transverse sections of lumbar spinal cords from day 11 fetuses. (a) Uninfected control fetus. Note proliferating neuroepithelial cells (NE) surrounding the lumen (L) and postmitotic neurons in the ventrolateral horns (V). (b and c) Mother infected with *ts*22 on day 8 of pregnancy. The neural tube has failed to close dorsally in panel b (arrows) and has been disrupted ventrally in panel c (arrowhead); the tissues otherwise appear normal. Toluidine blue was the stain used. Bar, 100 μm.

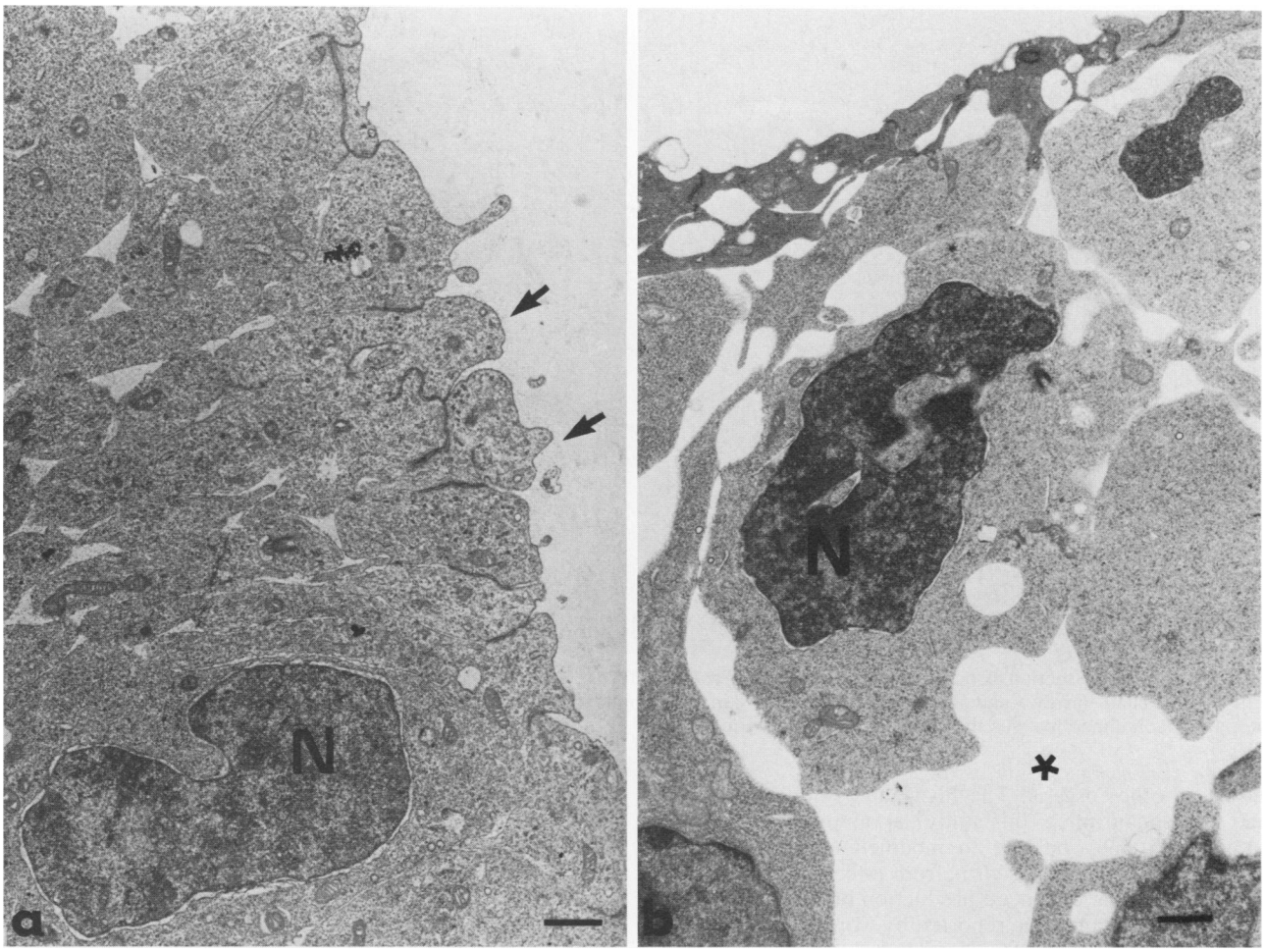

FIG. 6. Electron micrographs of apical ends of neuroepithelial cells of day 11 fetuses. (a) Uninfected control fetus; note pseudopodial processes (arrows), nucleus (N), and lumen (L). (b) Mother infected with *ts*22 on day 8 of pregnancy. Note the irregularly shaped nucleus with condensed chromatin (N), enlarged intercellular space (*), and abnormal pseudopodia. Bar, 1 μm.

day 10 and examined by IGSS and in situ hybridization. Both of these fetuses showed positive staining by in situ hybridization in parts of the fetus other than the CNS (although they were negative by IGSS).

The studies described above involved infection at day 10 of pregnancy, since this was the time at which the highest proportion of externally visible defects could be induced (15). We also used IGSS to examine fetuses from triplicate mothers infected at days 8 and 12 of pregnancy. IGSS-positive fetuses were detected at day 17 only (Table 1). The pattern of staining was similar to that produced following infection at day 10; most viral antigen was concentrated in the skin and cartilaginous plate, with less in the CNS.

Neural tube morphology. Formation of the neural tube was complete by day 11 of pregnancy in control fetuses (Fig. 5a).

Twelve fetuses derived from three females infected with *ts*22 on day 8 of pregnancy were examined at day 11. No histological abnormalities could be detected in two. Three had gross neural tube abnormalities consisting of dorsal open defects in two (Fig. 5b) and a ventral defect in one (Fig. 5c). In others, the neuroepithelium had many abnormal nuclei and mitotic figures, increased vascularization, abnormal pseudopodia, and enlarged intercellular spaces (Fig. 6).

Ten fetuses from three mothers infected on day 8 of pregnancy with *ts*22 were examined on day 14. One was dead, and no histological abnormalities were detected in the other nine. Ten fetuses from three mothers infected with *ts*22 on day 8 of pregnancy were examined on day 17. Of these, four fetuses were dead. All six surviving fetuses had patent central canals in the spinal cord (Fig. 7a). This contrasted with the normal temporary obliteration of the central canals seen in similarly aged control fetuses (Fig. 7b). Additional spinal cord lesions in five of the six surviving fetuses included an enlarged pool of postmitotic neurons in the dorsal gray matter and widening of the intercellular spaces in the white matter.

Ten fetuses from four mothers infected with A7 on day 8 of pregnancy were examined on day 11. Of these, six showed severe neural tube abnormalities, including ventral disruptions and distorted lumens. Many abnormal mitotic figures were present in the neuroepithela.

DISCUSSION

We have shown that both the wild-type A7 strain of SFV and the *ts*22 mutant derived from it infect the CNS and fetuses of pregnant mice and both induce viremia. The neurotropism of *ts*22 for adult mice is surprising, since no detectable viral nucleic acid or antigen was present in the CNS of infected fetuses before day 17 of pregnancy, in

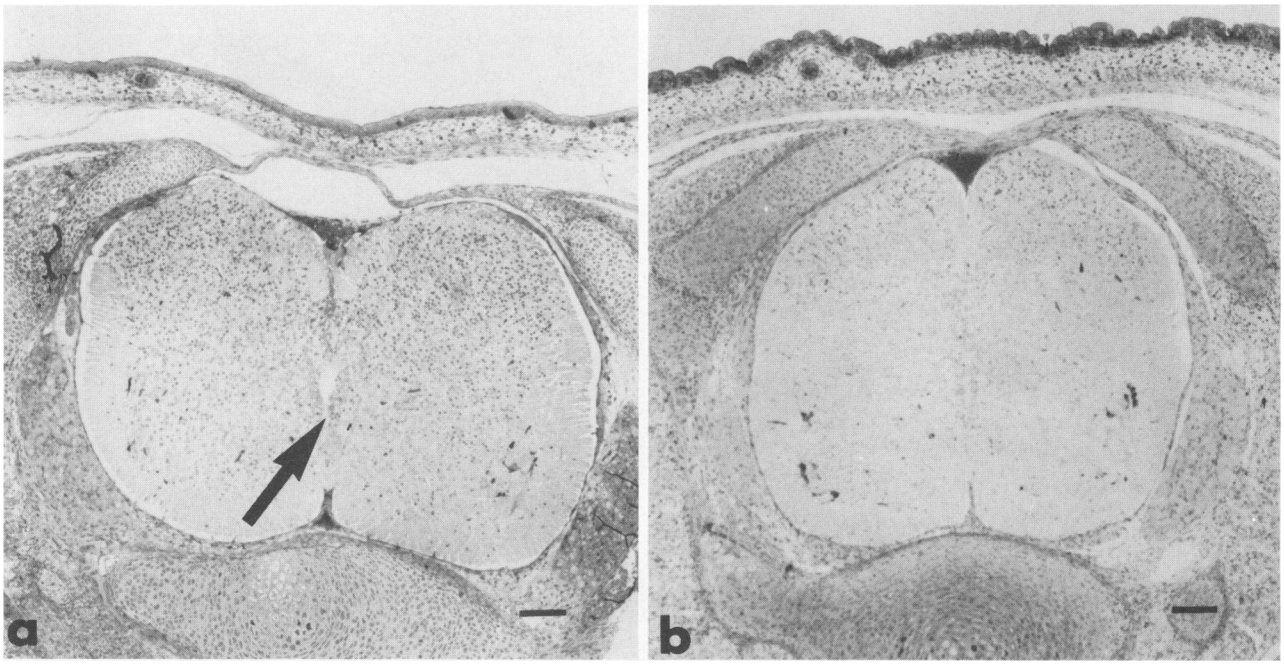

FIG. 7. Transverse sections from lumbar spinal cords of day 17 fetuses. (a) Mother infected with *ts*22 on day 8 of pregnancy. Note the patent central canal (arrow) and the poorly developed skin. (b) Uninfected control fetus. The central canal shows normal temporary obliteration. Toluidine blue was the stain used. Bar, 100 μm.

contrast to other organs. The high and prolonged viremia found in pregnant mice (this study) as opposed to nonpregnant mice (9) may be due to modified immunity during pregnancy or to release of virus from infected fetuses. It is consistent with the enhanced production of viral antigen by A7-infected pregnant mice reported by Milner et al. (18). In this study, the use of IGSS to detect viral infection was less sensitive than the use of nucleic acid probes; fetal infection before day 17 of pregnancy in live fetuses could generally be detected only with nucleic acid probes. The high titers of infectious virus found in pooled fetal extracts from pregnant mice probably reflect the virus contents of dead and necrotic fetuses; such fetuses were not subjected to IGSS or in situ hybridization.

One consequence of fetal infection by SFV is damage to the neural tube. For *ts*22, five cases of open neural tubes were detected which could not be associated with virus infection of neuroepithelial cells. In the case of the two ventral defects detected by use of sagittal sections in fetuses from mothers infected with *ts*22 at day 10 of pregnancy, we were able to show directly that neuroepithelial cells did not contain detectable viral nucleic acid, although other tissues in the fetuses did. For the three neural tube defects detected in fetuses after infection of the mother with *ts*22 at day 8 of pregnancy, transverse sections were used and prepared for light and electron microscopy. The method of fixation and embedding in these cases precluded the use of IGSS and in situ hybridization. However, neither virus particles nor cytopathic changes suggestive of virus infection (1, 3) were detected in neuroepithelial cells in these fetuses.

No open-neural-tube defects were detected at day 14 or 17. This could be due to abortion or resorption of affected fetuses. Dorsal neural tube defects, detected only following infection on day 8 of pregnancy, were probably due to failure of closure of the neural tube, whereas ventral defects could be due only to rupture of the neural tube once formed (21).

It is clear that neural tube damage induced by SFV in mouse fetuses may be associated with other fetal abnormalities; this is also the case with human spina bifida (7). Neural tube defects may be induced in developing mice by chemicals such as retinoic acid (13) and 5-bromodeoxyuridine (4). In this report, we show examples of virus-induced neural tube defects; such defects have been previously reported for infection of developing chicken embryos by influenza A virus and Newcastle disease virus (10–12, 20, 23). In the case of influenza virus, immunofluorescence studies showed that neural tube damage did not appear to be due to virus infection of neuroepithelial cells but was an indirect effect caused by virus infection of other tissues (10, 20). For Newcastle disease virus, damage appeared to be a direct result of virus infection (23). This study shows that viral nucleic acid and protein are absent from the CNS of *ts*22-infected fetuses before day 17 of pregnancy, although viral nucleic acid could be detected in mesenchymal cells adjacent to the developing CNS. Thus, damage to the neural tube is more likely to be due to changes induced by infection of these cells rather than a direct effect on the CNS.

Figure 8 summarizes the postulated mechanism of fetal pathogenicity for A7 and the *ts*22 mutant derived from it on the basis of this study, our previous studies (1, 8, 9, 15), and studies performed by other workers (17, 18). For A7, fetal infection occurs rapidly following maternal infection, viremia, and seeding of the placenta (9, 17). Although maternal immunity to A7 may be induced (1, 18) and transferred across the placenta to the fetus (1), this does not occur rapidly enough to prevent extensive fetal damage by A7. Since *ts*22 is lethal for neonatal mice (9) and some fetuses, the complete sparing of other fetuses and the induction of teratogenic effects may be related to variation in the rapidity of transfer of maternal immunity. The teratogenic effects induced by *ts*22 appear to result from rapid infection of mesenchymal cells; other cells, such as cells of the CNS,

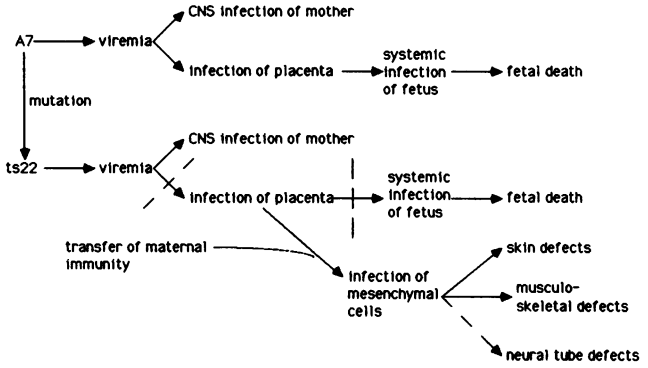

FIG. 8. Postulated mechanisms of pathogenicity of A7 and the *ts*22 mutant derived from it for fetal mice. Dotted lines indicate partial defects in fetal pathogenicity produced by the *ts*22 mutation. The dotted arrow represents an indirect effect; the solid arrows represent direct effects.

either are not susceptible at this stage of development or are infected more slowly. Direct damage to mesenchymal cells results in induction of musculoskeletal and skin defects (15), whereas induction of open-neural-tube defects, as shown in this study, results indirectly from damage to such cells.

Although virus-induced teratogenicity and abortion are medical and veterinary problems, good laboratory models for their analysis are few. In this study and in our previous work (1, 8, 9, 15), we have established SFV infection of laboratory mice as one such model.

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