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Placental and Fetal Alterations Due to Venezuelan Equine Encephalitis Virus in Rats

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Histopathological changes in the placentas, embryos, and fetuses of rats inoculated intraperitoneally with the virulent Guajira strain of Venezuelan equine encephalitis virus were studied by light microscopy and immunoperoxidase methods. Rats inoculated before day 15 of pregnancy showed necrosis and hemorrhages in the embryonic disks. Swelling of cytoplasm and nuclear pyknosis of cyto- and syncytotrophoblastic cells were noted as early as 2 days after inoculation. During weeks 1 and 2 of pregnancy, death of the embryos was always observed 3 to 4 days after Venezuelan equine encephalitis virus inoculation. Placental and fetal damage varied among the specimens. In rats 18 days pregnant and sacrificed 2 days after inoculation, there were some viable fetuses; the placentas showed inflammatory reactions in the mesometrial and decidual vessels. Other rats sacrificed at 3 to 4 days after inoculation showed large placental infarcts with fetal death. Viremia peaked during day 2 after inoculation. Immunoperoxidase stains demonstrated viral antigens present in the decidua, myometrium, and cyto- and syncytotrophoblastic cells. These experiments provide additional data regarding the pathogenesis and structural damage in the placental and fetal tissues caused by Venezuelan equine encephalitis virus.

Transplacental transmission of rubella, as well as Western equine encephalitis, togaviruses has been convincingly demonstrated in humans (1, 22). Cases of massive cerebral necrosis is newborn children were attributed to maternal infection with Venezuelan equine encephalitis (VEE) virus during the second and third trimesters of pregnancy (25). In spite of these reports, the evidence linking human congenital malformation to equine encephalitis infection is still inconclusive (1, 22, 25).

Experimental transplacental transmission of Japanese B encephalitis virus in swines (5) and of St. Louis encephalomyelitis (2) and VEE (23) viruses in mice have been reported. One of the difficulties encountered in studying the intrauterine effect of lethal strains of equine encephalitis is that the infected rodents rapidly become ill and die; however, rats are relatively resistant to VEE virus when the infecting virus is administered in moderate doses (13). We have recently described the survival of rats after VEE virus infection with the virulent Guajira strain, using the intraperitoneal route; central nervous system alterations in those animals were considered to be a sequela of the acute infection (11). This experimental model led us to examine the pathogenesis of intrauterine infection in Sprague-Dawley rats. This work was undertaken to examine the morphological changes and virological behavior in the placentas and in fetuses of rats inoculated intraperitoneally with Guajira strain of VEE virus.

The experiments described provide additional insights regarding the pathogenesis and structural damage to fetal tissues and placenta caused by VEE virus.

MATERIALS AND METHODS

Virus and virus assay. The origin, passage history, and stock preparation of VEE virus (Guajira strain, IVIC-PH 117S) were described previously (11, 12). The virulent Guajira strain was isolated in 1962 from a human patient during a VEE epidemic in the northwestern part of Venezuela (21). The virus was used after two passages in BHK-21 cells followed by three passages in suckling mouse brain.

Virus infectivity was assayed in Vero cells by a plaque method, utilizing an agarose serum-free overlay (4).

Animals and experimental design. Eighty-three Sprague-Dawley rats approximately 100 days old were used in these experiments. The date of pregnancy was established by mating males with female rats during their estrus and by microscopic observation of the vaginal contents (Table 1).

Animals were inoculated intraperitoneally with 200 plaque-forming units (PFU) of VEE virus at 5, 12, 15, 18, and 20 days of pregnancy. Another group of rats were inoculated intraperitoneally at 15 and 16 days of

TABLE 1. *Virological, gestational, and morphological data*

Inoculation dose of VEE virus, Guajira strain (PFU)	No. of inoculated animals	Gestational age at inoculation (days)	Gestational age at sacrifice (days)	Pathological findings
200	10	3	5	Death and hemorrhagic resorption of all embryos; necrosis in
	5	3	6	embryonic disks; hemorrhages and leukocytic infiltrates
	5	9	12	Death of all embryos; necrosis, hemorrhages, leukocytic
	5	10	12	infiltrates of the uterus and embryonal tissues
	6	12	15	
	2	12	15	
	5	13	15	
	2	12	18	Death of all embryos with hemorrhages and resorption;
	2	12	20	inflammatory changes in the decidua
	5	15	18	Death of all fetuses; smaller fetal size and some necrosis;
	10	16	18	inflammatory infiltrates, hemorrhages, and infarcts in
	5	16	20	placental tissues; early inflammation in mesometrial and
	10	17	20	decidual vessels; later hemmorhages and infarcts in placentas;
	5	18	20	necrosis of fetuses; some fetuses alive at sacrifice
2,000	2	15	18	Morphological changes similar to those described above, but
	2	16	20	more severe; at 4 and 5 days after inoculation all fetuses
	2	16	21	dead; larger necrosis and infarcts in placenta

pregnancy with 2,000 PFU of VEE virus. After brief anesthesia with ether, the rats were sacrificed 2, 3, and 4 days after inoculation. The abdominal wall was opened and the uterus was examined. During weeks 1 and 2 of pregnancy, the uterine horns of the rats sacrificed at 5, 12, and 15 days of pregnancy were sectioned and fixed in 10% Formalin for histological examination. Fresh samples were stored in the freezer for virological studies. During days 18 and 20 of pregnancy, the fetuses and placentas were separated; some of them were frozen for virological studies, and others were fixed in 10% Formalin for histological purposes. There were approximately 438 embryos and fetuses.

Blood samples taken from 25 rats at 2, 3, and 4 days after inoculation were also titrated for VEE virus to establish the viremia. The segments of the uteri were fixed in 10% Formalin, and samples of the fetuses with the placentas were dehydrated and embedded in paraffin for histological examination. Hematoxylin and eosin, Masson trichrome, Wilder reticulum, and periodic acid-Schiff stains were performed.

Immunoperoxidase. The paraffin sections, 4 to 6 μm thick, were warmed in an oven at 60°C for 0.5 h. The sections were deparaffinized in xylene and ethanol at 100 and 95%, respectively. Endogenous peroxidase activity was blocked by incubating the sections for 20 min in a solution of methanol-hydrogen peroxide (1:4, vol/vol). Sections were washed in distilled water and incubated for 15 min with normal swine serum to eliminate background staining. The slides were placed in petri dishes at room temperature and covered with VEE virus rabbit antiserum for 30 min at 1:250 and 1:500 dilutions. A tris(hydroxymethyl)aminomethane (Tris)-saline buffer solution was used as the control. Then the sections were washed again with Tris-saline and incubated in peroxidase-antiperoxidase complex (1:100 dilution). The sections were rinsed in Tris-saline and flooded with 6 mg of 3,3-diaminobenzidine tetrahydrochloride diluted in 10 ml of Tris-saline containing 3 drops of 3% hydrogen peroxide. After incubation for 4 min, the sections were rinsed again in distilled water and counterstained with hematoxylin.

RESULTS

Light microscopic examination of the uterus at days 5 and 12 of pregnancy was performed 2 and 3 days after inoculation with VEE virus.

Histological sections revealed focal necrotic changes or hemorrhages and necrosis of the embryonic disk, with swelling of the cytoplasm and nuclear pyknosis of cyto- and syncytiotrophoblastic cells (Fig. 1). During weeks 1 and 2 of pregnancy, death of the embryos was always observed 3 to 4 days after VEE virus inoculation. In some animals sacrificed 6 to 8 days after VEE virus inoculation, necrosis and hemorrhage with fetal resorption was present. Necrotic changes made it impossible to separate embryos and placentas for virological studies.

Rats inoculated on days 15 and 16 of pregnancy showed death and necrosis of fetuses. Pathological changes in the placenta varied from one fetus to another (Fig. 2). The size and gross appearance of the fetuses varied depending upon the time of examination (days after inoculation). Some fetuses were necrotic. Others showed diminished size and weight when compared with control fetuses of the same gestational age. Histopathological changes varied from extensive

areas of necrosis to congestion and some perivascular hemorrhage in the central nervous system. A correlation between the inflammatory and hemorrhagic changes in the placenta with the necrotic alterations of the fetus was always established.

In rats 18 days pregnant, sacrificed 2 days after inoculation, some fetuses were alive, and the placentas showed only inflammatory changes in the mesometrial or decidual vessels; other placentas at 3 to 4 days after inoculation showed extensive infarcts with massive necrosis of fetal tissues.

Virological studies determined that maternal viremia peaked during day 2 after inoculation.

Rats in day 18 of pregnancy, 3 days after inoculation, showed virus titers in the blood of ca. 10^4 PFU/ml, whereas in the placenta titers ranged from 10^4 to 10^6 PFU/g of tissue. The virus titers in the corresponding fetuses were ca.

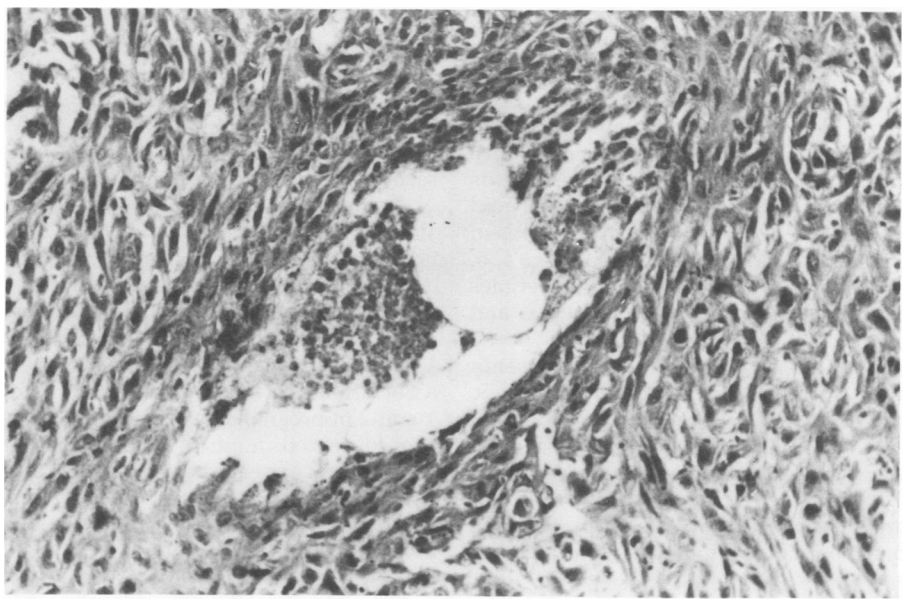

FIG. 1. *Necrosis of the embryonic disk, with swelling of the cytoplasm of cytotrophoblast and nuclear pyknosis. Hematoxylin and eosin; ×100.*

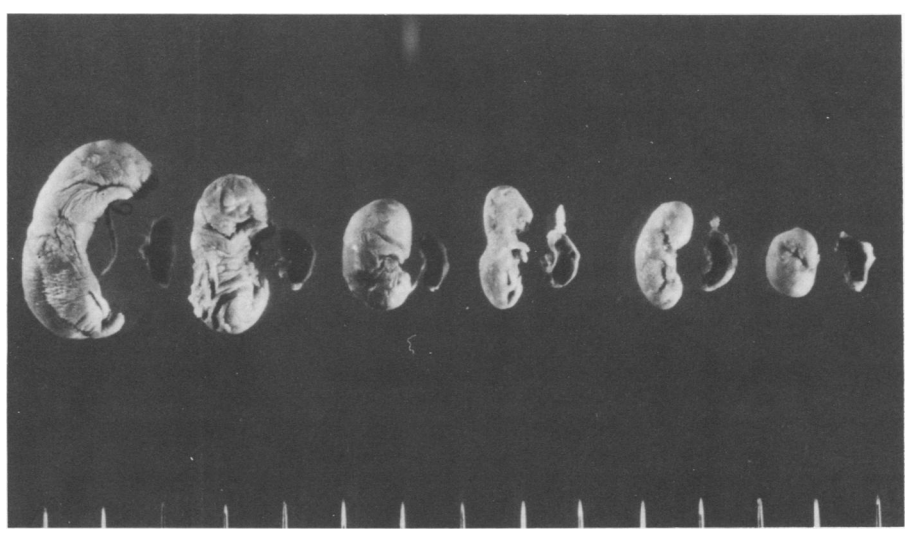

FIG. 2. *Gross alterations with various degrees of necrosis of the fetuses and placentas from a rat, 18 days pregnant, 3 days after inoculation with VEE virus. Hematoxylin and eosin; ×100.*

10^4 to 10^5 PFU/g of tissue. One day later, on day 4 after inoculation, blood titers had fallen to $<10^1$ PFU/ml, whereas the titer in the placentas and fetuses had increased up to ca. 10^7 and 10^5 PFU/g, respectively.

Histopathological changes in the placentas and fetuses examined on days 2, 3, and 4 post-inoculation in rats 18 days pregnant revealed a direct relationship between the placental alterations and the degree of necrotic changes observed in the fetuses. Early lesions were located in the mesometrial uterine arteries during day 2 post-inoculation, and inflammatory changes were noted in the wall of decidual vessels (Fig. 3). Congestion, edema of the intima, and infiltration with polymorphonuclear leukocytes and mononuclear cells in the wall of the vessels were conspicuous. At days 3 and 4 after VEE virus inoculation, necrosis of decidual cells, fibrinoid changes in the wall of the vessels, and angiitis in decidual arteries were also observed (Fig. 4, 5, and 6). Focal hyaline thrombi in the arterioles and decidual veins, as well as in the veins and capillaries of the chorionic plate, were noted (Fig. 7 and 8). Areas of necrosis in the placenta varied from focal alterations in the chorionic villi and within the decidua and trophoblastic cells to extensive hemorrhagic infarcts (Fig. 9). When the virus inoculum was increased from 200 to 2,000 PFU, gross and histological alterations were more conspicuous, and they occurred in a shorter period of time. Results of the immunoperoxidase technique also demonstrated virus antigens to be present in the sections of placentas examined 2, 3, and 4 days after inoculation. A positive staining reaction was initially present in decidual perivascular cells in the myometrium. Sections of placentas examined 3 and 4 days after inoculation showed very intense stained cells in the myometrium, decidua, and cyto- and syncytotrophoblastic zones (Fig. 10).

DISCUSSION

Experimental studies to determine the intrauterine effects of togavirus infection are of great importance, particularly since minimal brain damage due to prenatal exposure to VEE and St. Louis encephalomyelitis viruses has been recognized (2, 23). The report of massive necrosis of the brain in newborn children whose mothers suffered VEE virus infection (25, 26) has not been experimentally reproduced to the best of our knowledge; however, our results indicate that the placenta is a favorable site for replication of VEE virus. In the rats inoculated with VEE virus, transplacental infection occurred in association with maternal viremia. During weeks 1 and 2 of pregnancy, severe detrimental effects were observed in embryos. Since the incidence of stillbirths appeared to be dependent upon the gestational age at the time of infection, we focused our attention on the alterations of the placenta and fetus during week 3 of pregnancy.

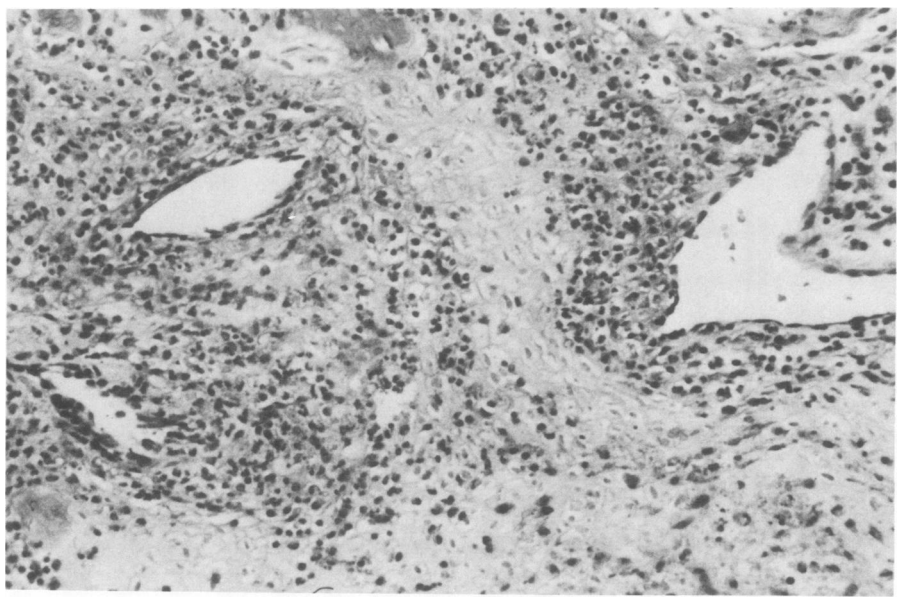

FIG. 3. *Inflammatory changes in the mesometrial arteries during day 2 post-inoculation with VEE virus. Hematoxylin and eosin; ×100.*

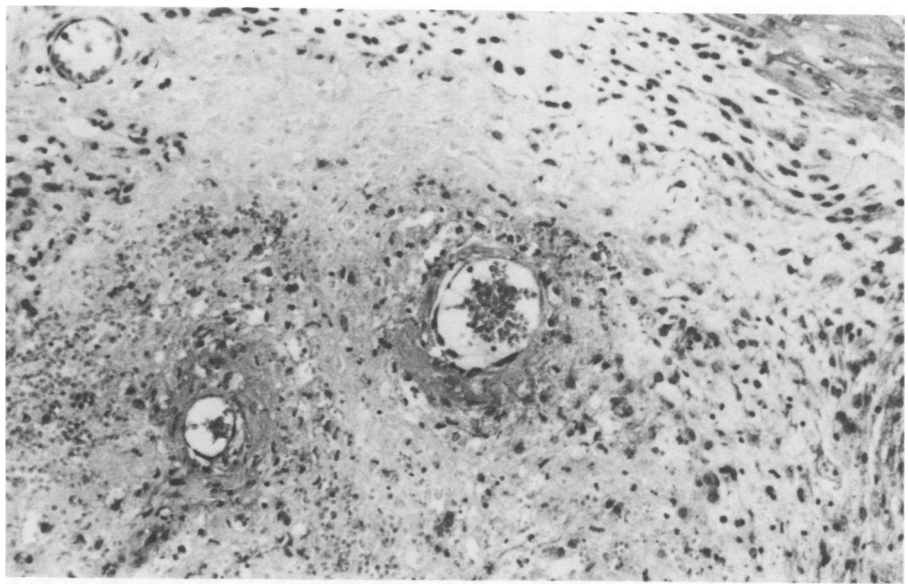

FIG. 4. *Fibrinoid necrosis of mesometrial arteries during day 3 after VEE virus inoculation. Hematoxylin and eosin; ×100.*

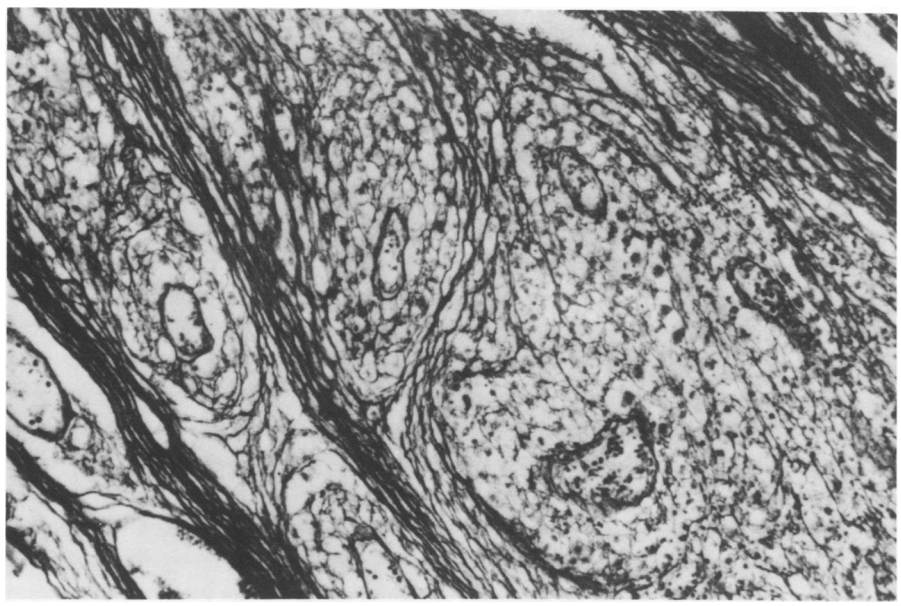

FIG. 5. *Reticulum framework surrounding decidual cells in the placenta of a control animal. Wilder reticulum; ×25.*

Wenger reported abortions in women suffering from VEE virus infection during the first trimester of pregnancy (26), whereas during the third trimester, necrosis of the fetal brain was demonstrated (25). Our experimental model revealed a similar situation regarding the fetal lesions of Sprague-Dawley rats.

Histological evidence of necrosis and focal thrombosis in the decidual and placental vessels as early as day 2 after intraperitoneal inoculation with VEE virus were parallel to active replication of VEE virus in the placenta. In the course of days 3 and 4 after inoculation, there was necrosis of the epithelium of the chorionic villi and thrombotic changes in the capillaries as well as in the large vessels of the placenta. Placental

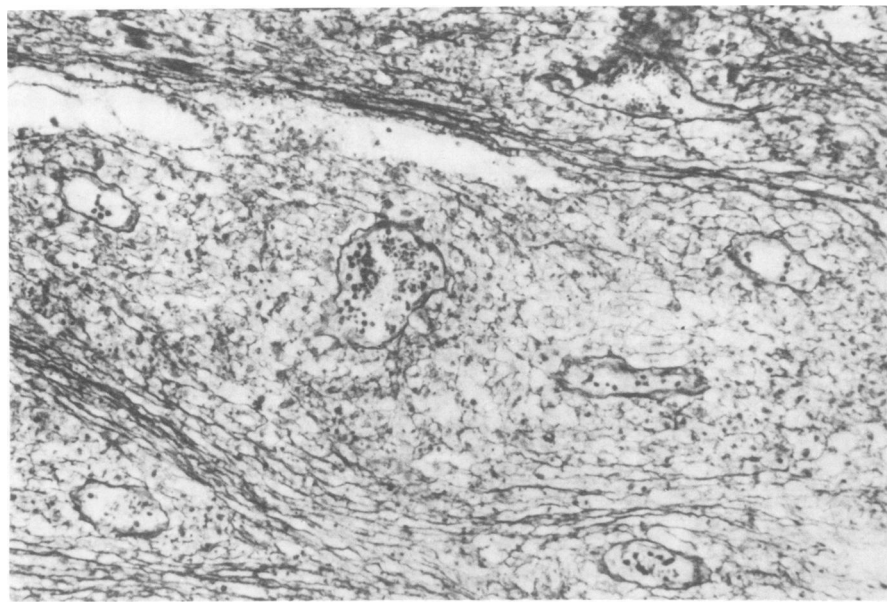

FIG. 6. *Focal disruption of the endothelial lining and distortion of the peridecidual reticulum framework in the placenta of an infected rat, 2 days after inoculation. Wilder reticulum; ×25.*

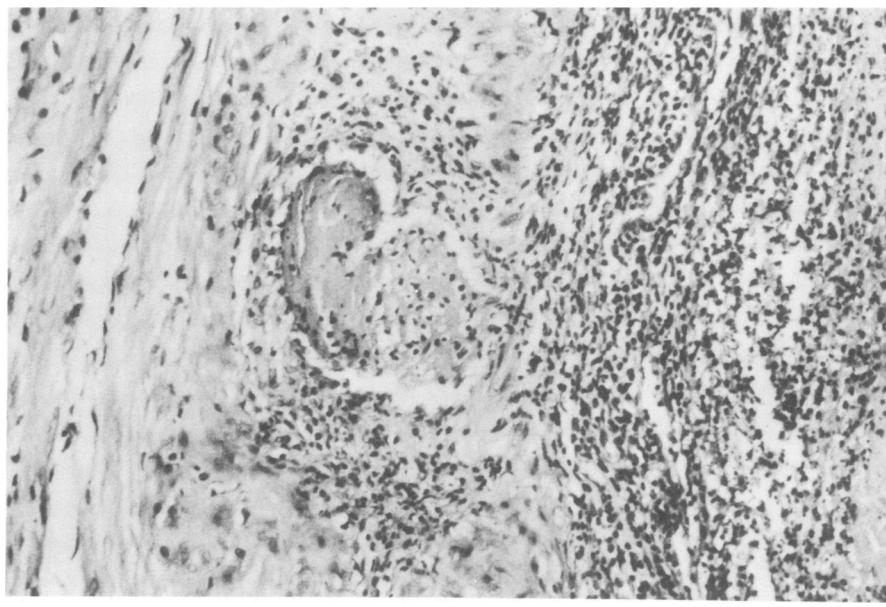

FIG. 7. *Large hemorrhagic infarction of the placenta at 4 days post-inoculation with VEE virus. Hematoxylin and eosin; ×25.*

infarcts varying in size were also present. Similar findings have been reported in the human placenta during rubella virus infection (1, 9, 18, 19, 24). The pathology of congenital infection with rubella virus is based on the embolization of pieces of necrotic placental vascular endothelium (9, 18, 19, 24). Persistent vascular endothelial damage has been shown in the brain of children with congenital rubella syndrome (20).

It is important to consider the vulnerability of decidual vessels at the time of viremia because vascular changes seemed to be the main factor

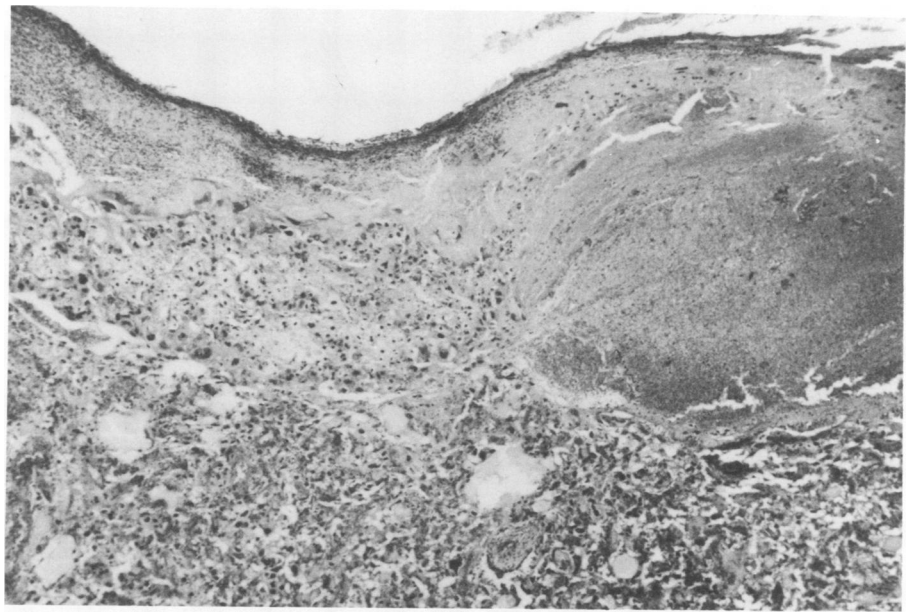

FIG. 8. *Hyaline thrombus in the wall of a vein at the chorionic plate, 3 days after VEE virus inoculation. Hematoxylin and eosin; ×100.*

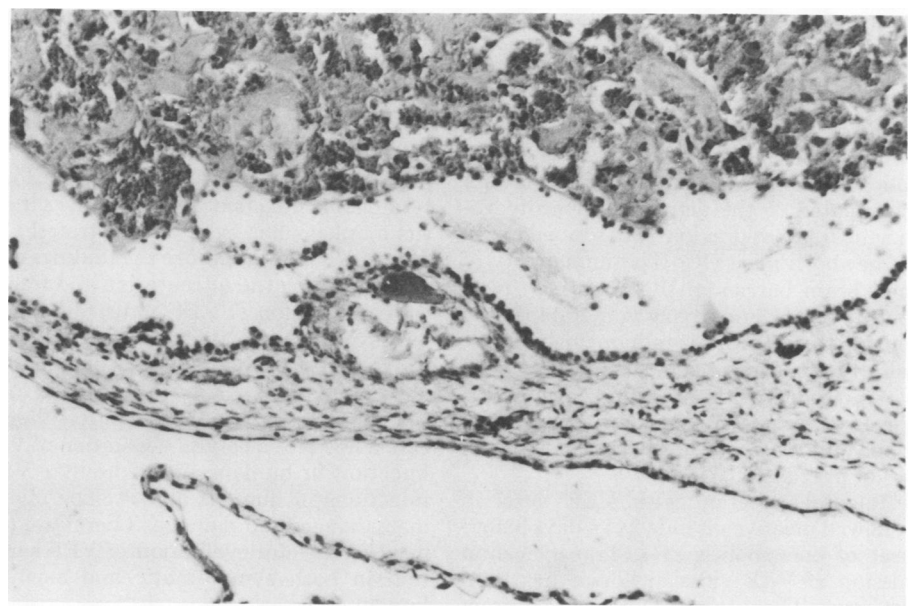

FIG. 9. *Modest angiitis and well-organized thrombus in a decidual artery, 4 days after inoculation with VEE virus. Hematoxylin and eosin; ×100.*

involved in the damage of the fetus. It is also possible that VEE virus crosses the placenta to replicate in the fetus, producing severe necrotizing changes in the brain. Arteriolar lesions in the mesometrial vessels of guinea pigs during high-altitude pregnancy have been considered to be related to the trophoblastic syncytial lining of the maternal vascular channels which in areas invade the decidual vessels (7). Although invading trophoblastic cells play the role of endothelial cells, it is possible that syncytial cells may be more easily damaged by hypoxia as well as

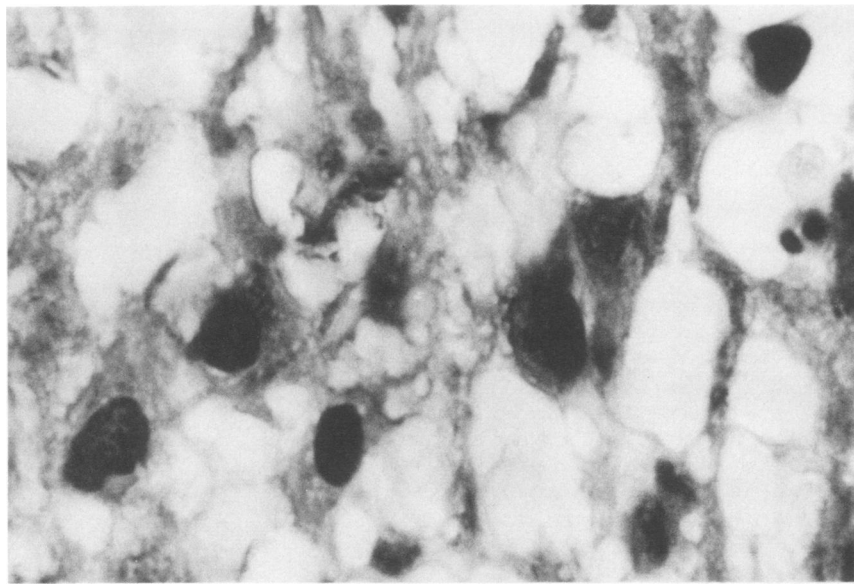

FIG. 10. *Two days after inoculation with VEE virus, the placenta shows cells surrounding the decidual vessels, stained positively. Immunoperoxidase stain; ×500.*

by VEE virus infection. Replication of virus in the vascular endothelium and decidual cells will induce necrosis, with cell lysis and release of large quantities of viral particles into the bloodstream. Immunoperoxidase stain confirmed the replication of VEE virus in the decidual and trophoblastic cells. Inflammatory changes during replication of VEE virus in the cells are probably related to the activity of acid hydrolytic enzymes, as was previously described in the brain of newborn mice (10). The immaturity of the blood brain barrier in the fetus may be a contributory factor underlying vulnerability.

In spite of the difficulties in inducing congenital malformations in mice (23) and rats with VEE virus, studies with St. Louis encephalitis virus revealed brain damage and neurological alterations in mice whose mothers were infected on day 8 of pregnancy (3).

Experimental infection with VEE virus in horses showed massive viremia 2 to 4 days before the onset of encephalitis (14). Transplacental transmission of VEE virus in horses has been demonstrated. The virus was recovered from fetal blood and organs, whereas no virus was isolated from the maternal blood, which contains neutralizing antibodies (15).

Rubella and VEE viruses are closely related members of the Togaviridae family. Pathological and virological evidence of intrauterine damage induced by VEE virus is similar to that induced by rubella virus infection (1, 9, 18, 24).

Electroencephalographic abnormalities and cerebral dysfunctions in children who suffered VEE virus infection have been reported (16). That clinical evidence was considered to be the counterpart of the central nervous system alterations observed as sequelae of VEE virus infection in rats (11). Causes of autism and behavioral abnormalities have also been reported in children with congenital rubella (6, 8). The strong teratogenic effects of VEE vaccine virus in rhesus monkeys has been demonstrated (17). This fact adds some support to the findings of Wenger (25) regarding the intrauterine and transplacental transmission of VEE virus. We believe that our experimental findings may provide some insights into the pathogenesis of equine encephalitis togavirus intrauterine infection in humans (22, 25, 26). The findings reported herein indicate a need for a careful evaluation of VEE virus infection in humans. It seems that VEE virus infections in humans and in experimental animals induce fetal damage. Therefore, there is a need for careful evaluation of VEE virus infection in both symptomatic and asymptomatic human cases.

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LITERATURE CITED

1. **Alford, C. A., F. A. Neva, and T. H. Weller.** 1964. Virologic and serologic studies of human products of conception after maternal rubella. N. Engl. J. Med. **271:**

1275-1281.
2. **Anderson, A. A., and R. P. Hanson.** 1970. Experimental transplacental transmission of St. Louis encephalitis virus in mice. Infect. Immun. **2**:320-325.
3. **Andersen, A. A., and R. P. Hanson.** 1975. Intrauterine infection of mice with St. Louis encephalitis virus: immunological, physiological, neurological, and behavioral effects on progeny. Infect. Immun. **12**:1173-1183.
4. **Bergold, G. H., and R. Mazzali.** 1968. Plaque formation by arboviruses. J. Gen. Virol. **2**:273-284.
5. **Burns, K. F.** 1950. Congenital Japanese B encephalitis infection in swine. Proc. Soc. Exp. Biol. Med. **75**:621-625.
6. **Chess, S.** 1971. Autism in children with congenital rubella. J. Autism Child. Schizophr. **1**:33-47.
7. **Delaquerrière-Richardson, L., and E. Valdivia.** 1967. Effects of simulated high altitude on pregnancy. Arch. Pathol. **84**:405-417.
8. **Desmond, M. M., G. S. Wilson, and W. M. Verniaud.** 1970. The early development of infants with congenital rubella. Adv. Teratol. **4**:39-63.
9. **Driscoll, S. G.** 1969. Histopathology of gestational rubella. Am. J. Dis. Child. **118**:49-53.
10. **García-Tamayo, J.** 1971. Acid phosphatase activity in mouse brain infected with Venezuelan equine encephalomyelitis virus. J. Virol. **8**:232-241.
11. **García-Tamayo, J., G. Carreño, and J. Esparza.** 1979. Central nervous system alterations as sequelae of Venezuelan equine encephalitis virus infection in the rat. J. Pathol. **128**:87-91.
12. **García-Tamayo, J., and J. Esparza.** 1978. Importancia de la respuesta celular en el fenómeno encefalítico inducido por el virus de la encefalitis equina venezolana. Patologia (Mexico City) **16**:215-231.
13. **Jahrling, P. B., A. DePaoli, and M. C. Powanda.** 1978. Pathogenesis of a Venezuelan encephalitis virus strain lethal for adult white rats. J. Med. Virol. **2**:109-116.
14. **Johnson, K. M., and D. H. Martin.** 1974. Venzuelan equine encephalitis. Adv. Vet. Sci. Comp. Med. **18**:79-116.
15. **Justines, G., H. Sucre, and O. Alvanez.** 1980. Transplacental transmission of Venezuelan equine encephalitis virus in horses. Am. J. Trop. Med. Hyg. **29**:653-656.
16. **León, C. A., R. Jaramillo, S. Martínez, F. Fernández, H. Teller, B. Lasso, and R. DeGuzmán.** 1975. Sequelae of Venezuelan equine encephalitis in humans: a four year follow up. Int. J. Epidemiol. **4**:131-140.
17. **London, W. T., N. H. Levit, S. G. Kent, V. G. Wang, and J. L. Sever.** 1977. Congenital cerebral and ocular malformations induced in Rhesus monkeys by Venezuelan Equine Encephalitis virus. Teratology **16**:285-296.
18. **Menser, M. A., and R. D. K. Reye.** 1974. The pathology of congenital rubella: a review written by request. Pathology **6**:215-222.
19. **Ornoy, A., S. Segal, M. Nishmi, A. Simcha, and W. Z. Polishuk.** 1973. Fetal and placental pathology in gestational rubella. Am. J. Obstet. Gynecol. **116**:949-956.
20. **Rorke, L. B., and A. J. Spiro.** 1967. Cerebral lesions in congenital rubella syndrome. J. Pediatr. **70**:243-255.
21. **Sellers, R. F., G. H. Bergold, O. M. Suárez, and A. Morales.** 1965. Investigations during Venezuelan equine encephalitis outbreaks in Venezuela 1962-1964. Am. J. Trop. Med. Hyg. **14**:460-469.
22. **Shinefield, H. R., and T. E. Townsent.** 1953. Transplacental transmission of Western equine encephalomyelitis. J. Pediatr. **43**:21-25.
23. **Spertzel, R. O., C. L. Crabbs, and R. E. Vaughn.** 1972. Transplacental transmission of Venezuelan equine encephalomyelitis virus in mice. Infect. Immun. **6**:339-343.
24. **Toundry, G., and O. N. Smith.** 1967. Fetal rubella pathology. J. Pediatr. **68**:867-879.
25. **Wenger, F.** 1967. Necrosis cerebral masiva del feto en caso de encefalitis equina Venezolana. Invest. Clin. **8**: 13-31.
26. **Wenger, F.** 1977. Venezuelan equine encephalitis. Teratology **16**:359-362.