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Congenital Abnormalities in Newborn Lambs After Infection of Pregnant Sheep with Akabane Virus

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Akabane virus (a Bunyavirus) has been associated with epizootics of congenital deformities in cattle, sheep, and goats. Experimental studies using mouse-adapted virus inoculated intravenously into pregnant sheep gave an inapparent infection. Neutralizing antibodies were detected on day 5, and peaks in the titer were seen at days 10 and 48. Ewes infected at day 30 to 36 of pregnancy produced five (31% incidence) deformed lambs. Sera from four of these possessed neutralizing antibodies to Akabane virus before ingesting colostrum. Two lambs had arthrogryposis, hydranencephaly, kyphosis, scoliosis, and brachygnathia; one had micrencephaly; and the other two had porencephaly. The two lambs with arthrogryposis and hydranencephaly also had extensive lesions in other tissues. In the spinal cord there was a marked decrease in the number of ventral horn neurones and a depletion of myelin. Skeletal muscles showed marked atrophy. The medulla of the thymus possessed large Hassall's corpuscles and a reduced number of thymocytes in the cortex. It would appear that the pathogenic effects of Akabane virus are related to the gestational age (30 to 36 days) at which the fetus is infected. Akabane virus can now be included in the growing list of teratogenic viruses and provides an interesting system for studying such congenital diseases.

Epizootic and sporadic outbreaks of congenital abnormalities characterized by arthrogryposis (AG), hydranencephaly (HE), or both of these (AG/HE), and micrencephaly (ME) have been reported in cattle, sheep, and goats in Australia (2, 7, 8, 24), Israel (13, 15), and Japan (11, 12, 14).

Since epizootic AG/HE was first described by Blood (2) and Whittem (24), various causes have been suggested, including genetic factors, teratogenic chemicals or toxins, vitamin or mineral deficiencies, and infectious agents. Recently, Miura et al. (14) and Hartley et al. (9) found neutralizing antibodies to Akabane virus in sera collected from calves with congenital AG/HE before they suckled colostrum. As antibodies do not normally cross the placenta in ruminants and the young receive antibody from their mothers at ingestion of colostrum, their presence in the sera of fetuses or neonates is considered a good indication of in utero infection (21). It would therefore appear that Akabane virus is a likely cause of epizootic congenital AG/HE in cattle.

Akabane virus is a member of the Simbu serological subgroup (3) of the *Bunyaviridae* family (19) of arboviruses. It was first isolated from the mosquitoes *Aedes vexans* and *Culex tritaeniorhynchus* in Japan by Oya et al. (18); in Australia it has been isolated from the biting midge *Culicoides brevitarsus* (3). This paper reports the results of infecting pregnant ewes with Akabane virus. It demonstrates that transplacental transmission of the virus occurs over a limited gestation range and results in the production of congenital abnormalities.

MATERIALS AND METHODS

Virus. The B8935 strain of Akabane virus (3) isolated from *C. brevitarsis* was used throughout this study. The virus had successively received three passages in mice, followed by one passage in cattle and two further passages in mice. A clarified 10% suspension of brain from infected suckling mice was used for experimental infection. It had a virus titer of between 10^6 and $10^{7.4}$ 50% minimum lethal doses (MLD_{50})/ml when assayed in suckling mice. For neutralization tests, the virus was used after a further two to four passages in cell culture.

Cell cultures. The virus was grown in either cell lines of African green monkey kidney (Vero) cells (23) or baby hamster kidney (BHK21) cells (5). The Vero cells were grown in medium 199 containing 5% fetal calf serum (growth medium) and maintained in medium 199 containing 0.2% bovine serum albumin and buffered with 15 mM HEPES *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Calbi-

ochem, San Diego, Calif.) at pH 7.5 (maintenance medium).

Microtiter neutralization test. The method used (A. J. Della-Porta, M. D. Murray, and D. H. Cybinski, Aust. Vet. J., in press) for the assay of serum neutralizing antibodies to Akabane virus is described briefly. Twofold serial dilutions of heat-inactivated (56°C for 30 min) sera were made in maintenance medium in transfer plates using microdiluters (Cooke Engineering, Alexandria, Va.). A total of 12 to 25 50% tissue culture infective dose units of virus was added to each well, and the transfer plates were incubated at room temperature (20°C) for 1 h. The incubated serum-virus mixtures were transferred to the flat-bottom tissue culture trays (Cooke Engineering, Alexandria, Va., or Falcon Plastics, Los Angeles, Calif.) containing maintenance medium (0.2 ml/well) and a confluent monolayer of Vero cells (seeded the previous day at 4×10^4 cells/well). The test was read after 3 to 4 days of incubation at 37°C in an atmosphere of 5% CO_2 in air. Antibody titers are expressed as the reciprocal of the initial dilution of serum that, after mixing with virus, neutralized the virus infectivity in 50% of the wells inoculated at the dilution (20).

Experimental procedure. Merino and Border Leicester-Merino cross ewes at various stages of pregnancy (see Table 1) were infected by an intravenous inoculation of Akabane virus (10^6 to 10^7 MLD_{50}) suspended in 1 to 2 ml of Hanks balanced salt solution (pH 7.6) containing 0.2% bovine serum albumin. Seven Merino ewes were given 2 ml of clarified 10% normal suckling mouse brain in Hanks balanced salt solution containing 0.2% bovine serum albumin by intravenous inoculation at day 30 to 36 of pregnancy. Before infection or inoculation all of the ewes were bled, and the sera were harvested and assayed for neutralizing antibody to Akabane virus. After infection both groups of ewes were kept in insect-proof isolation units until parturition. They were bled at weekly intervals, and the sera were harvested and stored at −20°C until assayed for neutralizing antibody. Observations were made and the temperatures of the ewes were recorded twice daily for the first 2 weeks and then daily thereafter. Before parturition each ewe was fitted with a brassiere to prevent lambs suckling colostrum.

At autopsy within hours of birth each lamb was examined for gross lesions, and a wide range of tissue samples were taken for virus isolation and histological examination. The blood collected was heparinized for virus isolation and that for serological examination had no anticoagulant added.

Collection of samples for virus isolation. The following specimens were examined: heparinized blood, thyroid, thymus, lung, heart, lymph nodes (mediastinal, mesenteric, and retropharyngeal), liver, spleen, kidney, adrenal, urine, muscle, brain (cerebrum, cerebellum, and medulla), and spinal cord (cervical, thoracic, and lumbar). Ten percent suspensions of tissue samples were made in Difco heart infusion broth, pH 7.4, and inoculated immediately into litters of 1- to 3-day-old mice by the intracerebral route. The mice were examined daily and any showing signs of sickness were killed, their brains were removed, and suspensions prepared in broth were inoculated into further mice. If there were no signs of ill health within 10 days after inoculation, a suspension was made from a pool of brains from all of the mice in the litter and inoculated into additional mice. All samples were passaged two to four times in suckling mice before being discarded as containing no virus.

Collection of tissues for histopathology. Samples of thymus, lung, heart, liver, kidney, spleen, skeletal muscle, adrenal, and lymph nodes were fixed in Bouin solution, and brain and spinal cord were fixed in 10% neutral buffered Formol saline. All tissues were embedded in paraffin, and sections were stained with hematoxylin and eosin (H & E). Brain and spinal cord sections were also stained with Luxol fast blue.

RESULTS

Akabane virus infection in ewes. A total of 22 ewes at various stages of pregnancy (Table 1) were infected. None showed clinical signs of infection, and their body temperatures remained within the normal range for the duration of the experiment. Serum neutralizing antibodies against Akabane virus (Fig. 1, sample group) were detected as early as 5 days after infection. There was usually an initial rise in titer to a peak at 10 days, followed by a slight drop and a second peak 4 weeks after infection. There was a slight decline of antibody levels throughout the remainder of pregnancy. Fluctuations occurred in the titers, although tests were carried out simultaneously on all sera collected from each ewe. There was no difference in antibody responses between ewes producing deformed lambs and those producing normal lambs, irrespective of the stage of pregnancy at which the ewes were infected.

Results of lambing. All ewes inoculated with virus completed their pregnancies 140 to 148

TABLE 1. *Experimental infection of pregnant ewes with Akabane virus*[a]

No. of days pregnant	No. of ewes	No. of lambs born	No. of deformities
28	1	2	0
30–36	11	16	2 AG/HE 2 PE 1 ME
38–41	3	6	0
50	2	3	0
63–67	3	4	0
82	2	2	0

[a] Ewes were inoculated intravenously with 1 to 2 ml of Akabane virus suspension (10^6 to $10^{7.4}$ MLD_{50}/ml).

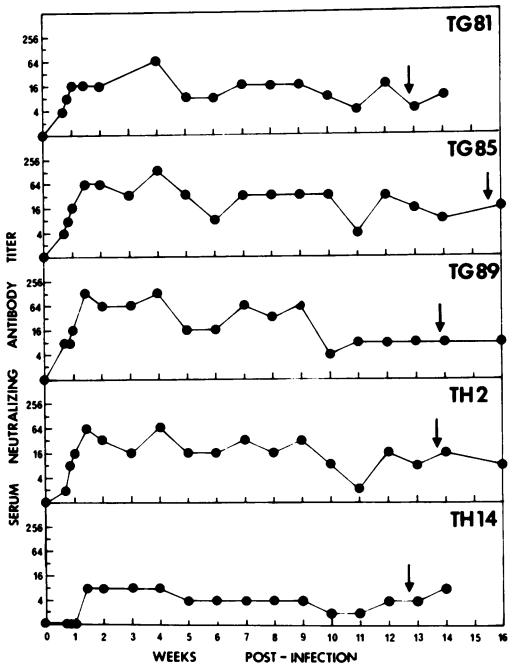

FIG. 1. *Serum neutralizing antibody responses in pregnant ewes after intravenous inoculation with Akabane virus ($10^{7.4}$ MLD_{50} doses). The ewes were infected at the following stages of pregnancy: TG81 at 63 days, TG85 at 36 days, TG89 at 50 days, TH2 at 50 days, and TH14 at 63 days. The time of birth of the lambs is indicated by the arrows.*

days after mating. A total of 33 lambs (11 sets of twins and 11 singletons) were born, of which 28 were normal (Table 1). Of the other five lambs, two had severe AG and were dead at birth, two had porencephaly (PE), and one had ME (Tables 1 and 2). One of the lambs with AG had a normal twin; the other had a twin with PE. The seven ewes in the control group inoculated with noninfectious mouse brain suspension completed their pregnancies, and each ewe had one normal lamb. Neutralizing antibodies to Akabane virus (Table 2) were present in the sera of four of the lambs with congenital deformities and in the serum of a normal (TG24F1) twin to one of the deformed lambs. Neutralizing antibodies were also present in the sera of two lambs from ewes inoculated on day 50 of pregnancy. Neutralizing antibodies were not detected in the serum of one of the lambs with PE (TG22F1), its twin, or a normal twin to the lamb with ME (TG85F1) (Table 2). No other lamb had serum neutralizing antibodies to Akabane virus. Virus was not isolated from any of the lambs.

Morbid anatomy. The two lambs with AG presented similar skeletal deformities: kyphosis, scoliosis, and brachygnathism (Fig. 2a, b, and d, respectively). There were marked changes in the skeletal musculature, including loss of muscle mass. Both carcasses had depleted fat deposits, especially in the area of brown fat. The skulls of both lambs appeared normal, but most of the forebrain had been replaced by membranous sacs of liquid and the structures remaining were not recognizable (Fig. 2c). The midbrain, cerebellum, and medulla oblongata were reduced in size, whereas, the spinal cord, which was reduced in size through the cervical region, tapered off sharply from the thoracic to lumbar region.

The two lambs with PE had large liquid-filled cavities in the subcortical areas of the cerebrum. The remainder of the brain and the spinal cords appeared normal. The remaining lamb with ME had difficulty walking and suckling. Its head was small, the bones of the skull were abnormally thick, and the cranial cavity was reduced in size when compared with its twin (Fig. 3a.) The brain was markedly smaller than that of the twin mainly due to a reduction in the size of the cerebral hemispheres (Fig. 3b). No macroscopic lesions were noted in any of the other 28 lambs or in the 7 control lambs.

Histopathological examination. In the lambs with AG and HE the meninges consisted of fused membranes with focal areas of necrotic debris and fibrous thickenings. The spinal cord was reduced in size in both lambs, and there

TABLE 2. *Presence of neutralizing antibodies to Akabane virus in precolostrum sera of lambs born to ewes infected with Akabane virus*[a]

Stage of pregnancy when infected (days)	Ewe identification		Deformity in lamb at birth	Titer of antibodies
30	TH4	F1[b]	AG/HE	2
		F2	PE	2
33	TG22	F1	PE	<1
		F2	Normal	<1
34	TG24	F1	Normal	4
		F2	AG/HE	8
36	TG85	F1	ME	2
		F2	Normal	<1
50	TG89		Normal	8
50	TH2	F1	Normal	16
		F2	Normal	<1

[a] Only ewes that gave birth to deformed lambs or had antibody-positive lambs are shown in this table. Sera from all other lambs born to infected ewes (Table 1) were free from antibody to Akabane virus.

[b] F1, fetus 1; F2, fetus 2.

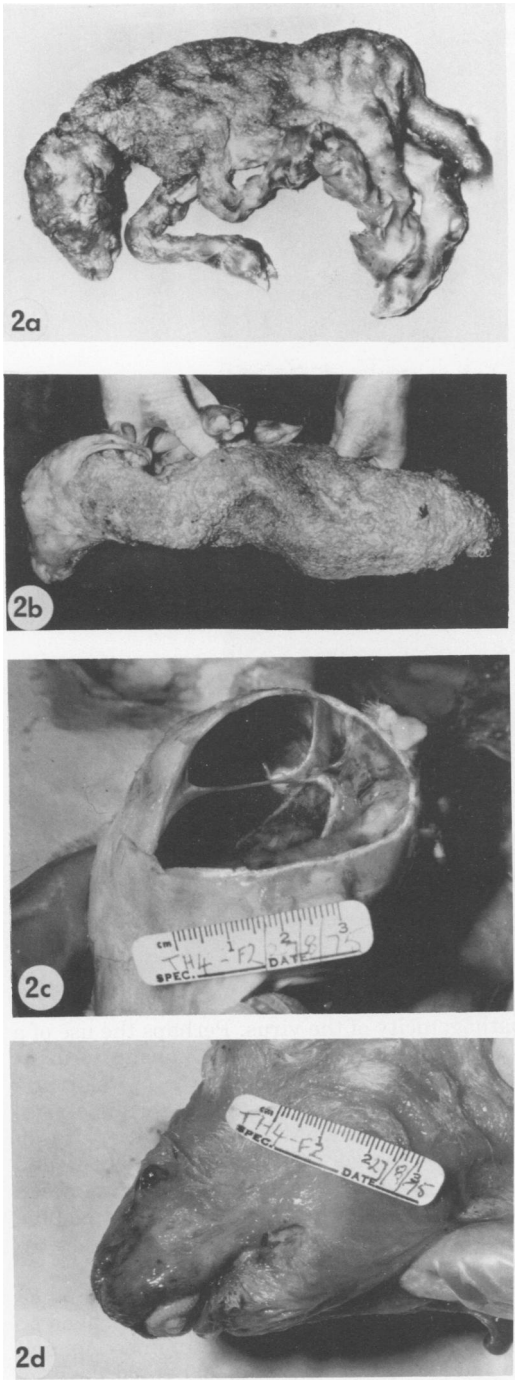

FIG. 2. *Congenitally deformed lambs produced by intravenous inoculation of pregnant ewes, at 30 to 36 days gestation, with Akabane virus. (a) Side view of lamb showing AG and kyphosis. (b) Dorsal view of a lamb showing severe scoliosis. (c) Lamb with HE. The removal of the calvarium showed an almost complete replacement of the brain by cerebrospinal liquid. The liquid has been aspirated and the brain remnants are visible. (d) Muzzle of a lamb showing brachygnathia.*

was a marked reduction in the extent of myelination of the ventral cord and a reduction in the ventral horn neurones (Fig. 4), many of which often exhibited degenerative changes. Small mineralized plaques were present beneath the meninges of the spinal cord (Fig. 5).

Of the other tissues examined the skeletal muscles, especially those of the limbs, showed the most marked lesions. Muscle bundles varied in size, and areas of atrophy and degeneration were present (Fig. 6). Elsewhere there was a loose arrangement of mixed tissues, consisting of some adipose cells and fibrous tissues among the muscle cells (Fig. 7).

In the medulla of the thymus, Hassall's corpuscles were prominent and contained much keratin and eosinophilic hyaline material (Fig. 8). The cortex was narrow and sparsely populated with thymocytes.

In the two lambs with PE, numerous small to large areas of cavitation (Fig. 9) and small malacic foci were present in the cerebral subcortex. There was very little evidence of an inflammatory reaction to these lesions.

The sections of the brain of the lamb with ME showed few changes that had not already been noted during the gross examination. There was a decrease in the parenchyma of the cortex of the cerebrum, but no degenerative changes were seen. The spinal cords of the lambs with

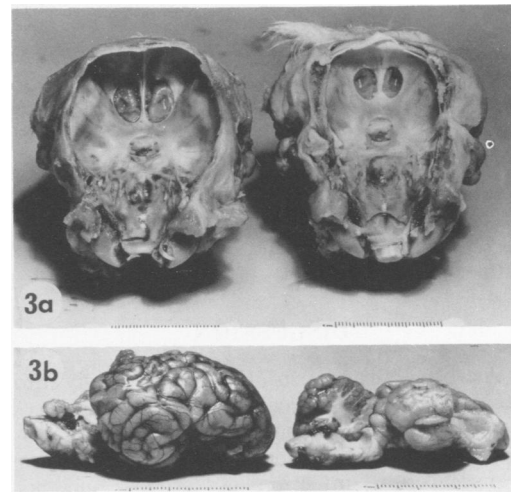

FIG. 3. *Normal lamb (left) its congenitally deformed twin (right), showing ME, produced by intravenous inoculation of a pregnant ewe, at 36 days of gestation, with Akabane virus. Comparison of (a) the cranial cavities and (b) the brains.*

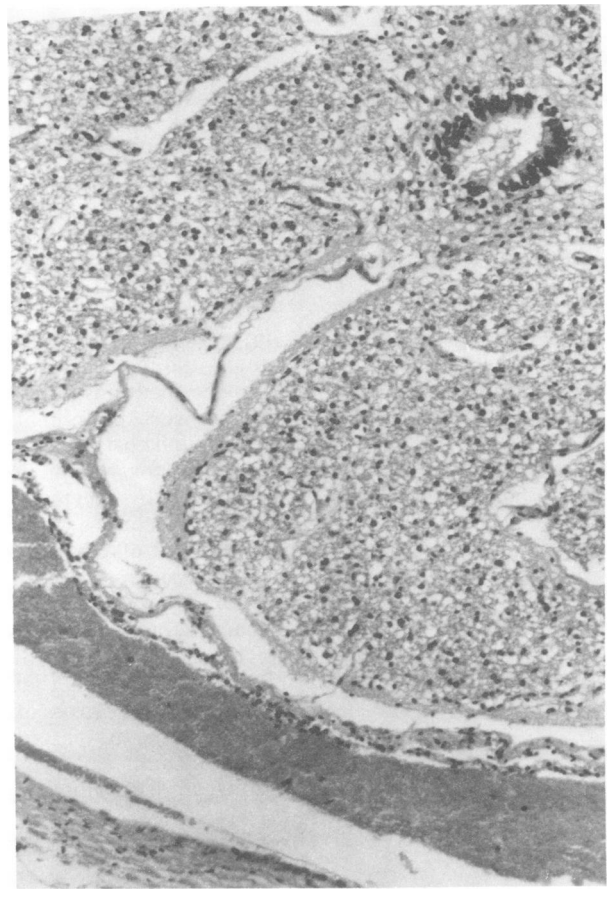

FIG. 4. *Spinal cord from a lamb with AG/HE, showing a marked decrease in ventral horn neurones and myelin. H & E stain. ×140.*

PE and the lamb with ME appeared normal, and no lesions were seen in other tissues examined. No microscopic lesions were noted in any of the remaining 28 lambs or in the control lambs.

DISCUSSION

Akabane virus inoculated intravenously into pregnant ewes produced an inapparent infection. The birth of abnormal lambs from a proportion of the infected ewes indicated that transplacental infection of fetuses had occurred, producing both macroscopic and microscopic changes. This diagnosis was supported by the demonstration of neutralizing antibodies in the precolostral sera of lambs from 4 (36%) of the 11 ewes inoculated between days 30 and 36 of gestation. It is not known whether the small proportion of ewes in which transplacental infection occurred is similar to the incidence under field conditions or whether passage of the virus in laboratory systems has reduced the pathogenicity of the virus. Perhaps the use of a low-passage field isolate of Akabane virus or the use of the virus after growth in its arthropod vectors may increase the proportion of fetal infections and congenital abnormalities. It is of interest, however, that when Anderson and Jensen (1) inoculated pregnant ewes between week 5 and 6 of gestation, with attenuated blue tongue virus, transplacental transmission occurred in only 30% of the ewes.

The pathogenic effects of Akabane virus on the developing ovine fetus appear to depend on the gestational age at the time of infection, and within narrow time limits (30 to 36 days) of gestation a wide range of pathological changes was noted. The variations ranged from AG and HE to PE and ME. Serological results showed that Akabane virus had also crossed the placenta in ewes inoculated with virus when pregnant for 50 days, but no lesions were noted in the lambs born to these ewes.

In the United States congenital defects in newborn lambs have been associated with vaccination of pregnant ewes with attenuated live blue tongue virus vaccine during weeks 5 and 6 of gestation (22). Osburn et al. (17), in a detailed study of the pathology of these congenital defects, inoculated blue tongue virus intramuscularly into fetal lambs in utero. They found that the type of malformations depended on the stage of development of the fetus. In lambs infected between days 50 and 58 of gestation, a severe necrotizing encephalopathy developed, which was seen as HE at birth, and lambs infected between days 75 and 78 developed a

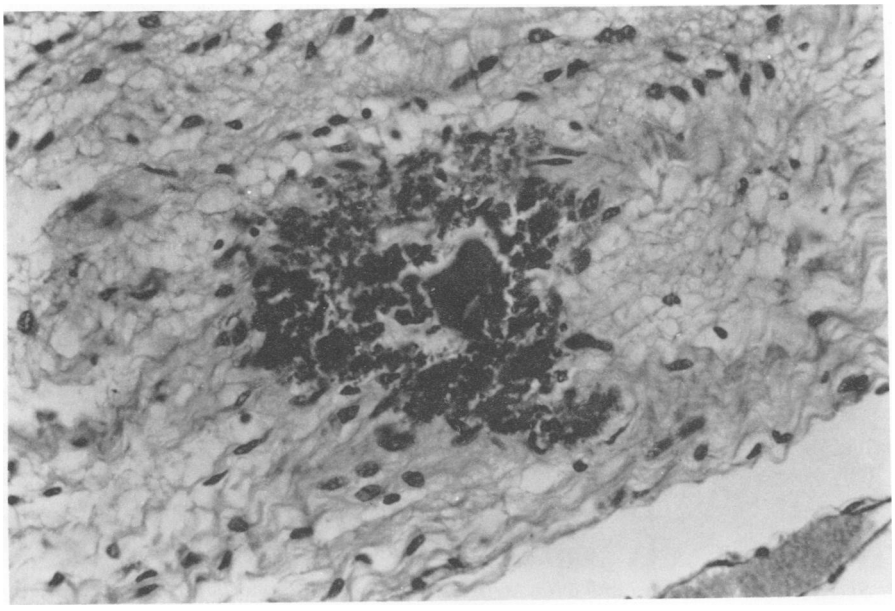

FIG. 5. *Spinal cord from a lamb with AG/HE, showing mineralized plaque in submeningeal area. H & E stain. ×350.*

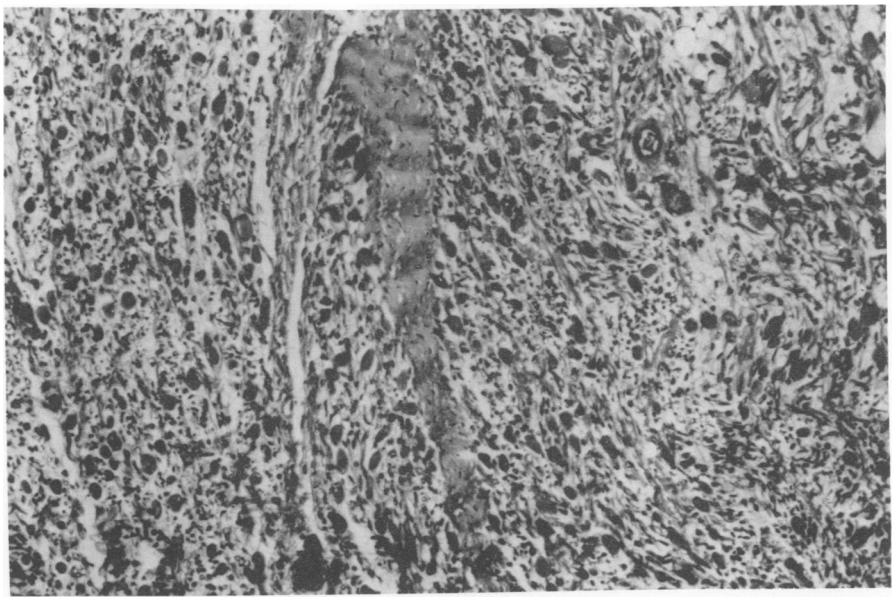

FIG. 6. *Skeletal muscle from a lamb with AG/HE, showing marked loss of structural form and the admixture of fibrous and adipose tissues. H & E stain. ×140.*

multifocal encephalitis and vacuolation of white matter, which appeared as porencephalic cysts at birth. Both of these types of lesions were seen in our series of lambs infected with Akabane virus, and as these occurred after infection over a limited gestational range it could be assumed that the type of lesion is dependent on the stage of development of the fetus.

Osburn et al (17) suggested that the severe necrotizing encephalopathy is limited to the very young fetus because of the presence of immature neural cells with an enhanced viral

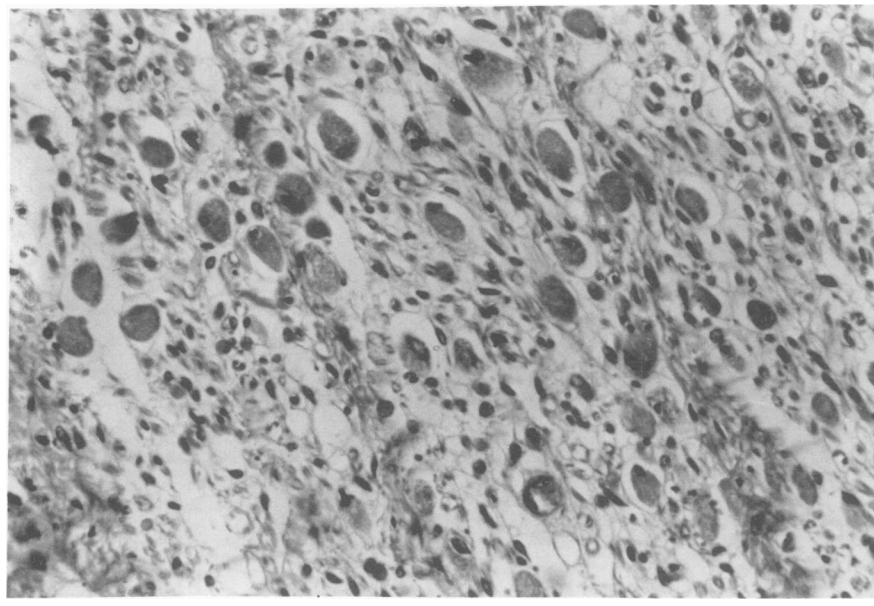

FIG. 7. *Skeletal muscle from a lamb with AG/HE, showing rounding of muscle cells, with adipose cells and fibrous tissue also present. H & E stain. ×350.*

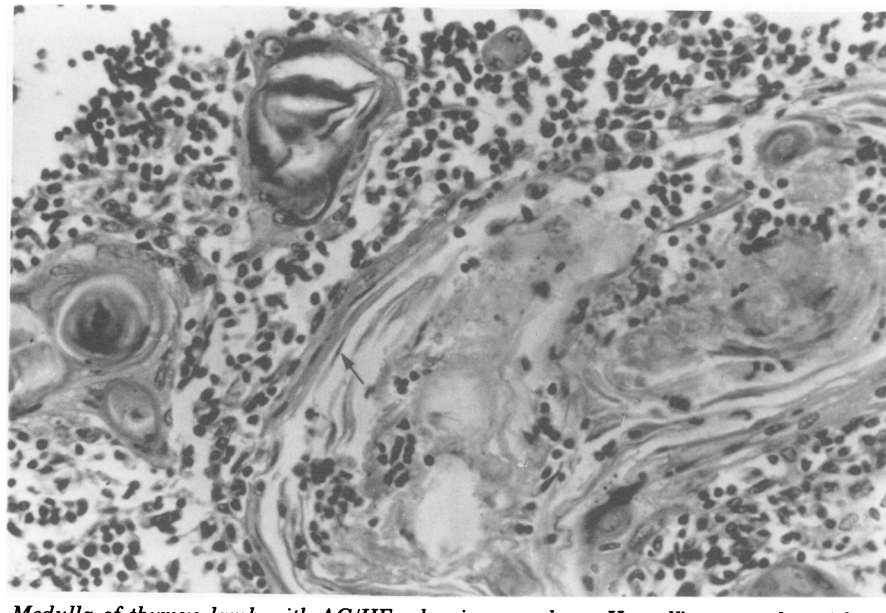

FIG. 8. *Medulla of thymus lamb with AG/HE, showing very large Hassall's corpuscles with prominent myoepithelial cells (arrow) and containing hyalin-like eosinophilic material. H & E stain. ×350.*

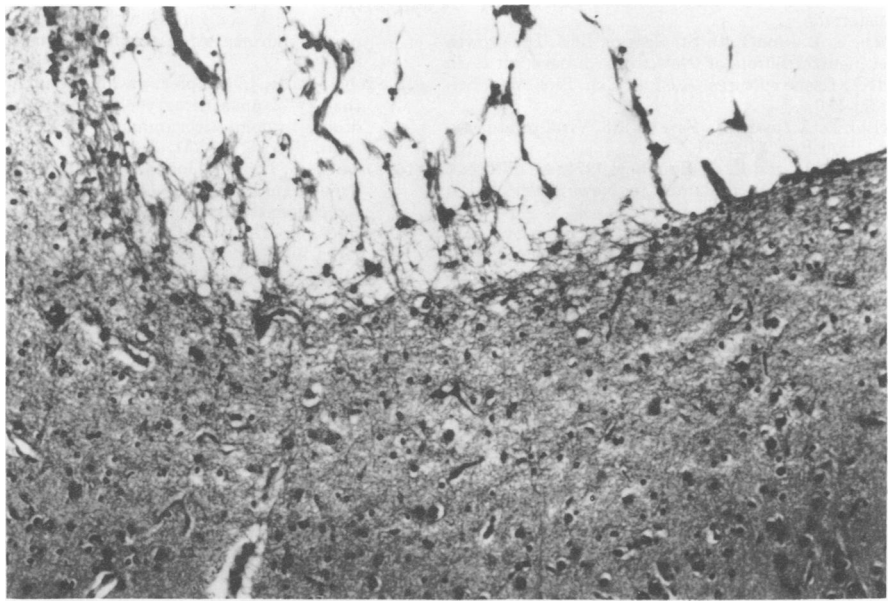

FIG. 9. *Lamb with PE, showing central area of cavitation surrounded by a zone of edema in the white matter of the cerebrum. Note appearance and lack of inflammatory cell response. H & E stain. ×140.*

susceptibility, combined with an inability to mobilize an effective immunological response. From our limited observations in Akabane disease in fetal lambs, it would appear that similar mechanisms also operate in this virus infection.

Neutralizing antibodies to Akabane virus were detected in the sera of the infected ewes as soon as 5 days after inoculation, and most ewes developed a biphasic antibody response (Fig. 1), the second peak being 4 weeks after infection. This probably represents a normal primary response of sheep to infection (10). The maintenance of antibody levels (Fig. 1), with subsequent fluctuations, could be related to the intermittent release of Akabane virus or related antigens and warrants a more detailed investigation.

The presence of neutralizing antibodies in the precolostrol sera of four out of five of the affected lambs would indicate that Akabane virus or viral antigens were present when the fetus became competent to produce antibody at about 60 to 70 days of gestation (4). Our inability to isolate the virus from the fetuses may have been due to the presence of this antibody, which could have eliminated or masked the virus. Immunoglobulins were detected in ovine fetuses infected with blue tongue virus as early as day 64 of gestation, but neutralizing antibodies to blue tongue virus were not detected until 122 days of gestation (16); it is possible that a similar response is produced to Akabane virus by the fetus.

Akabane disease in sheep provides an interesting model of a congenital infection, about which very little is known in regard to how the virus causes congenital abnormalities and how long the virus persists in the fetus. Studies of this disease in sheep also has direct relevance to understanding similar diseases caused by Akabane virus in cattle and goats. Such studies could also have much wider implications in determining the causes of mental retardation and congenital deformities in humans and various animal species (6). This study adds Akabane virus to the growing list of teratogenic viruses, and further studies are in progress to investigate the mechanism by which it causes congenital defects.

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