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Congenital Abnormalities in Newborn Calves After Inoculation of Pregnant Cows with Akabane Virus

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A fresh isolate of Akabane virus was inoculated intravenously into 11 seronegative pregnant cows at 62 to 96 days of gestation. Two of the cows were slaughtered 18 days post-inoculation, and the fetuses were examined; the remaining cows were allowed to give birth. All the inoculated cows developed viremia and neutralizing antibody for the virus, indicating that the cows were actually infected with the virus, although fever or any other clinical abnormalities were not noted. The virus further infected the fetuses. This was proved by virus isolation in one of the two fetuses from the slaughtered cows, and polymyositis was noted in both fetuses. Six of seven calves born alive had anti-Akabane antibody in their precolostral sera, indicating that in utero infection with the virus took place in these calves. Some of the in utero-infected calves demonstrated congenital abnormalities such as cerebral defect, hydranencephaly, and arthrogryposis. These findings provide additional evidence that Akabane virus is the etiological agent of epizootic abortion and congenital arthrogryposis-hydranencephaly syndrome in cattle.

Epizootics of abnormal deliveries such as abortion, stillbirth, premature birth, and calf deformities referred to as congenital arthrogryposis-hydranencephaly (AH) syndrome were observed among cattle in Japan during the summer through winter months of 1972-73 and 1973-74 (3-5, 9-11, 13-16, 20, 21, 24-28, 31). The central nervous systems of affected fetuses and newborn calves showed various degrees of damage, and, more importantly, inflammatory changes, particularly in cases occurring in the early stages of the outbreaks (13, 14, 21, 24, 27). This finding, together with the seasonal occurrence and the geographical distribution of cases, suggested an etiology of an infectious nature (26, 27). Our serological studies of the outbreaks strongly suggested that Akabane virus, a member of the Simbu group of arboviruses, was the etiological agent, since we could demonstrate a close correlation between the congenital AH syndrome of calves and antibody for Akabane virus in their precolostral sera and serological evidence for wide dissemination of Akabane virus among cattle in the epizootic areas during summer months in 1972 and 1973 (15, 20). This view was further corroborated by the isolation of Akabane virus from naturally affected fetuses (16).

Cases of a congenital AH syndrome have also been reported in cattle, sheep, and goats in Israel (19, 23) and in cattle and sheep in Australia (1, 2, 6, 7, 18, 32, 33). Recently, serological evidence for the etiological role of Akabane virus in congenital AH syndrome in cattle, sheep, and goats has been obtained in Australia (8) and Israel (12). Furthermore, congenital AH syndrome was reproduced by inoculation of pregnant sheep (Y. Hashiguchi et al., personal communication; 30) and pregnant goats (17) with Akabane virus.

In the present study, intrauterine infection of fetuses occurred after intravenous inoculation of pregnant cows with Akabane virus, and congenital abnormalities including AH syndrome were observed in infected fetuses.

MATERIALS AND METHODS

Virus. Strain OBE-1 of Akabane virus, at the primary passage level in baby mice, was used to inoculate pregnant cows. The strain was recovered from a naturally infected bovine fetus (16). To prepare the virus material for inoculation, the original infectious brain tissues of the fetus, which had been stored at −80°C, were used for intracranial inoculation of 1- to 2-day-old suckling mice. When the inoculated mice developed encephalitis, the brains were harvested and a 10% suspension was made with Eagle minimum essential medium containing 10% tryptose phosphate broth and 10% calf serum devoid of neutralizing (NT) antibody for Akabane virus. The suspension had a titer of 3.1×10^6 plaque-

forming units (PFU)/ml and was confirmed to contain Akabane virus by an NT test with specific antiserum. The material was stored at −80°C until used.

Inoculation of pregnant cows. Eleven pregnant cows, ten of Holstein-Friesian breed and one of Japanese Black breed, were inoculated with 5 ml of the Akabane virus suspension described above. The cows were 3 to 4 years old, and all were nulliparous and at 62 to 96 days of gestation when inoculated (Table 1). All the cows were confirmed to be negative for NT antibody against Akabane virus before virus inoculation.

Virus recovery. Blood samples from the inoculated cows and selected tissues, fluids, and blood from the fetuses were stored at −80°C and tested for the presence of Akabane virus by the plaque count method on HmLu-1 cell monolayers as described previously (16). Each 10% tissue suspension and undiluted blood and fluid specimens, all clarified by centrifugation, were inoculated in 0.2-ml amounts into two to four cell cultures, and the amount of virus was expressed in PFU.

NT test. All the specimens were stored at −20°C until used. The test was carried out by the microtiter method, using Vero cells and Akabane virus strain JaGAr39 (29) as described previously (15). For the test with colostrum, colostral whey prepared by the method of Mukkur and Froese (22) was used. The antibody titer was expressed as the reciprocal of the highest dilution preventing cytopathic effect of the virus.

RESULTS

Observations of cows. Clinical observation and blood count were made daily, and blood samples for detection of viremia were also taken daily for 7 days postinfection. The cows were tested weekly for serum NT antibody to Akabane virus.

Fever and any other clinical responses were not noted in any of the inoculated cows. Leukopenia was observed in 5 of the 11 cows (Table 1). Viremia was detected in all the inoculated cows at 1 to 6 days postinfection, persisting for 1 to 4 days. Virus titers ranged from $10^{0.1}$ to $10^{2.7}$ PFU/ml (Table 2). NT antibody to Akabane virus was produced in all the inoculated cows (Fig. 1). At the time of delivery, the cows had serum NT titers of 8 to 32, and their colostrum contained NT antibody in titers of 2 to 128 (Table 3).

Virus recovery from fetuses. Two of the 11 inoculated cows (no. 611 and 612) were slaughtered 18 days after intravenous inoculation with Akabane virus to investigate whether infection of the dams resulted in infection of the fetuses.

As mentioned earlier, although these cows did not develop fever, leukopenia, or any other clinical abnormalities after virus inoculation (Table 1), they developed viremia (Table 2) and NT antibody to Akabane virus (Fig. 1), indicating that they had actually acquired virus infection.

The fetuses obtained from these dams 18 days postinfection showed no gross pathological changes except subcutaneous hemorrhages in the head, neck, waist, and extremities. Histologically, polymyositis as observed previously in a natural case (16) was demonstrated in both fetuses, but no inflammatory changes were noted in the central nervous systems of the fetuses.

Selected tissues and other specimens from these fetuses were tested for virus. The intestinal tissues and pooled fetal membranes (amnion and allantois) of the fetus of cow 611 were shown to contain $10^{0.1}$ and $10^{1.9}$ PFU of virus per 0.1 g of tissue, respectively, but no virus was recovered from brain, spinal cord, lung, heart, liver, spleen, kidney, skeletal muscle, stomach, fetal and maternal placenta, cord blood, and

TABLE 1. *Experimental production of congenital abnormalities by inoculation of pregnant cows with Akabane virus*

Cow no.	Days of gestation when inoculated[a]	Fever	Leukopenia	Viremia	Duration of pregnancy[b] (days)	Gross findings of fetus or newborn calf
611	95	−	−	+	113	Subcutaneous hemorrhage
612	85	−	−	+	103	Subcutaneous hemorrhage
568	96	−	+	+	271	AH syndrome
608	82	−	+	+	286	Normal
618	87	−	+	+	286	Normal
619	76	−	+	+	268	Cerebral defect
620	62	−	−	+	103	Autolyzed
621	70	−	−	+	286	Normal
622	86	−	+	+	129	Mummified
625	78	−	−	+	278	Jaundice
631	92	−	−	+	271	AH syndrome

[a] Pregnant cows were inoculated intravenously with strain OBE-1 of Akabane virus.
[b] Cow 611 and 612 were slaughtered 18 days postinfection, and the other cows were allowed to give birth.

TABLE 2. *Viremia in inoculated cows*

Cow no.	Virus titer (log PFU/ml)						
	1[a]	2	3	4	5	6	7
611	0.7	–[b]	0.4	–	–	–	–
612	–	–	–	–	–	–	–
568	–	–	0.1	1.3	2.2	1.9	–
608	–	0.2	1.2	1.7	0.8	–	–
618	–	–	1.7	1.2	–	–	–
619	–	–	1.2	0.7	–	–	–
620	–	–	1.8	2.4	–	–	–
621	–	0.7	2.2	1.9	–	–	–
622	–	–	1.0	2.7	1.1	–	–
625	–	1.5	2.2	1.1	0.2	–	–
631	–	0.7	0.5	–	–	–	–

[a] Days after virus inoculation.
[b] –, Negative with undiluted blood specimen.

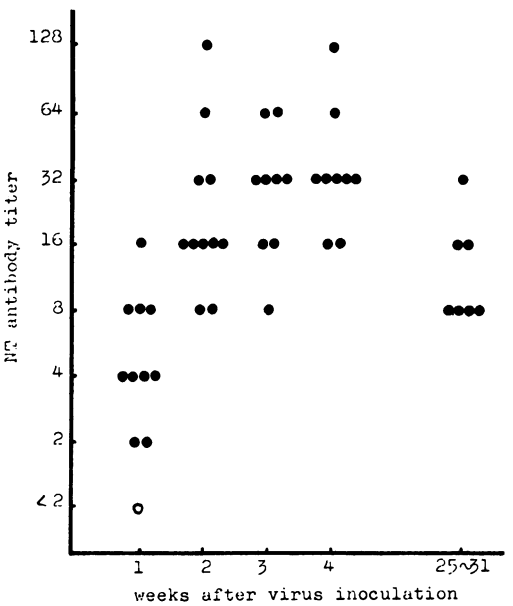

FIG. 1. *Production of NT antibody to Akabane virus in inoculated cows.*

amniotic and allantoic fluid. No virus was recovered from the fetus of cow 612. Umbilical cord serum samples from these two fetuses were negative for NT antibody to Akabane virus.

Congenital abnormalities in calves of infected dams. Nine of the 11 inoculated cows were allowed to give birth to investigate whether infection of the dams with Akabane virus induced congenital abnormalities of the fetuses as observed in natural cases. As mentioned earlier, the inoculated virus obviously succeeded in establishing infection in all the dams, since viremia and NT antibody production were demonstrated.

TABLE 3. *NT antibody to Akabane virus in sera of newborn calves before and after ingestion of colostrum, and in sera and colostrum of the dams*

Cow no.	NT antibody titer			
	Dam		Newborn calf	
	Serum	Colostrum	Precolostral serum	Postcolostral serum
568	8	64	16	16
608	8	32	<1	32
618	16	16	16	32
619	32	16	2	64
620	32	NT[a]	NT	NT
621	16	2	8	4
622	32	NT	NT	NT
625	8	16	2	2
631	8	128	16	32

[a] NT, Not tested.

Of the nine cows, cow 620, which was inoculated with virus at 62 days of gestation, delivered an autolytic fetus with hemorrhagic placenta 41 days postinfection. No virus was recovered from this fetus. Cow 622, which received the virus at 86 days of gestation, delivered a mummified fetus 43 days later (Table 1).

Three of the remaining seven dams (568, 619, and 631) gave birth to calves with congenital abnormalities (Table 1). The calf from cow 568 had defects in both the left and right temporal lobes of the cerebrum, hydrocephalus, and a minor twist of the right foreleg with erratic gait (Fig. 2). The calf of cow 619 had a slight defect in the left temporal lobe of the brain, but no other abnormalities. The calf of cow 631 had

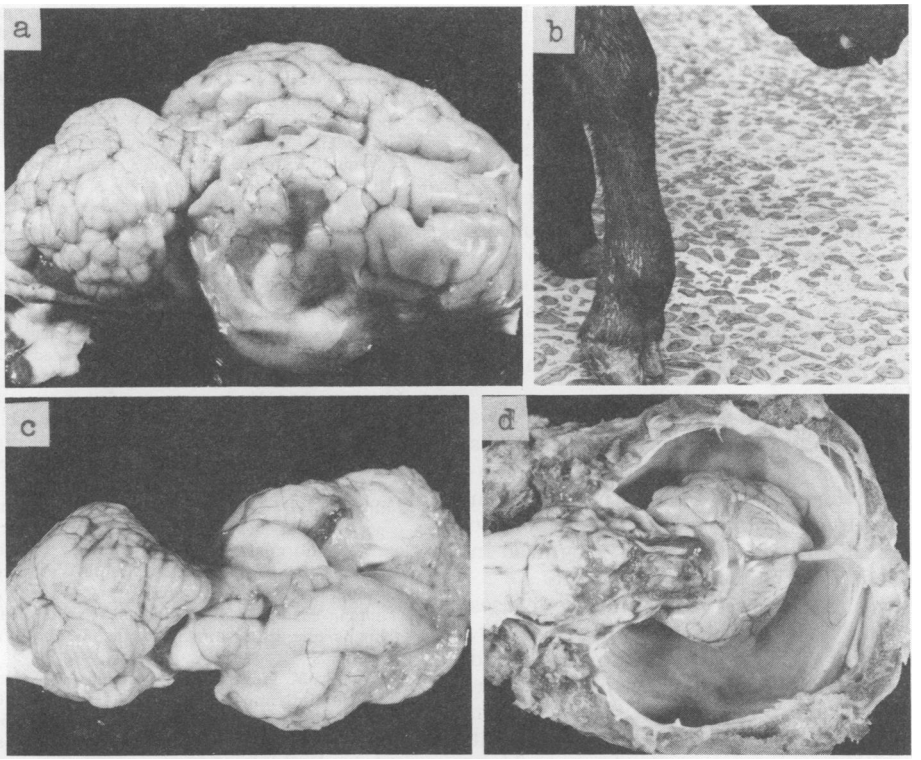

FIG. 2. *Macroscopic findings of congenital malformation in calves delivered from cows inoculated with Akabane virus. (a) Cerebral defect (no. 568); (b) minor twist of right front limb (no. 631); (c) cerebral defect (no. 631); (d) hydranencephaly (no. 631).*

hydranencephaly with a grossly defective brain, the cranial cavity being filled with about 50 ml of fluid (Fig. 2). The calf was blind and could not suckle, and the tongue was paralyzed. The calves of the remaining four dams had no congenital deformities, but one of them had juandice and was weak, its body weight at birth being only 27 kg. These calves with congenital deformities and the weak calf tended to be delivered somewhat earlier than normal calves (Table 1). The last three calves, those of cows 608, 618, and 621, were all delivered in term uneventfully and showed neither clinical manifestations nor gross pathological changes. Precolostral serum samples from these seven calves were tested for NT antibody for Akabane virus, and all but one were positive in titers of 2 to 16. The NT-negative calf (608) was healthy and became NT positive after ingestion of colostrum (Table 3).

DISCUSSION

In the present study the OBE-1 strain, a fresh isolate of Akabane virus from a naturally infected bovine fetus, induced intrauterine infection of fetuses when inoculated intravenously into seronegative pregnant cows. Some of the in utero-infected calves from these cows demonstrated congenital abnormalities as observed in natural cases of congenital AH syndrome. These findings provide additional evidence that Akabane virus is the etiological agent of epizootic abortion and congenital AH syndrome in cattle. As mentioned earlier, this view was first advanced by our serological studies on epizootics of the disease (15) and were further corroborated by the isolation of Akabane virus from naturally affected fetuses (16).

All the inoculated cows were considered to be actually infected with Akabane virus, since they developed viremia and NT antibody for the virus. The virus further infected the fetuses. This was proved by virus isolation 18 days after inoculation in one of the two fetuses. Of the seven calves born live from the remaining cows, six had NT antibody to Akabane virus in their precolostral sera. We believe this indicates that in utero infection with Akabane virus took place in these claves, since the passive transfer of maternal antibody into the fe-

tus does not occur in cows and the fetus develops the ability to produce antibody upon antigenic stimulation early in gestation (15). The congenital abnormalities observed in some of the in utero-infected calves were quite reminiscent of the AH syndrome in natural cases (13, 14, 21, 24, 27).

In the present study, the cows infected with Akabane virus developed viremia. This observation, together with the previous results indicating the occurrence of viremia and infection of the placenta in naturally infected cows (16), suggests that Akabane virus may infect the fetus through hematogenous infection of the placenta.

In none of the experimentally infected cows were fever or any other clinical abnormalities noted. This finding coincides with the observation that in the epizootics of abnormal births in 1972–74, no clinical abnormalities that could be related to abnormal births were recognized in the dams during their pregnancy (4, 15).

Polymyositis, as observed in the two fetuses examined 18 days postinfection in this study, was previously noted in a naturally infected fetus and proved by virus isolation and immunofluorescent staining to result from infection of muscle cells (16). These findings suggest that polymyositis may be an important cause of congenital deformities and muscular damages observed in natural cases, although these changes could also be sequelae of central nervous system involvement.

Further investigations are needed to elucidate the pathogenesis of fetal infection with Akabane virus.

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