アカバネ病生ウイルス予防液に対する妊娠めん羊の応答

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Responses of Pregnant Ewes Inoculated with Akabane Disease Live Virus Vaccine[1]

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Studies were made on the safety and immunogenicity of Akabane disease live virus vaccine (the TS-C 2 strain), developed to prevent cows from infectious abnormal birth caused by this virus, for pregnant ewes. In them, 12 ewes were inoculated subcutaneously with this vaccine at 30～37 days of pregnancy. As a result, viremia was detected in three of them. The neutralizing antibody titer was 2～128 three weeks later. To examine the effect of immunization by inoculation with vaccine, six of the twelve ewes (group A) 42 days after inoculation and four uninoculated control pregnant ewes (group C) were challenged by inoculation with the high virulent OBE-1 strain. After challenge viremia was negative in all the ewes of group A, but appeared in all the animals of group C. When four fetuses were collected from group C 14 days after challenge, the virus was recovered from several organs of each of them. No virus was recovered from any ewe of group A. On the other hand, antibody was detected in fetal serum from all the ewes, except one, of group A. It was negative in fetal serum from any animal of group C. Of the 12 experimental ewes, the other six (group B) were inoculated with the vaccine and subjected to no challenge inoculation. Of them, four gave normal birth, one was sacrificed at 144 days of pregnancy to collect twin fetuses, and the other one was sterile. All the newborn lambs and the twin fetuses were free from any abnormal change in body shape and brain. Antibody was found in their serum and the precolostral serum of six lambs. From these results, it was concluded that Akabane disease live virus vaccine could not be used for pregnant ewes on account of its poor safety on these animals.

The congenital arthrogryposis-hydranencephaly syndrome was prevalent in Japan over a period of 1972 to 1975. From the results of a seroepizootiological survey and the isolation of virus from bovine fetuses involved, it was demonstrated to have been caused by infection with Akabane virus (a member of the Simbu group of the Bunyaviridae).[5~8,10,13,15] Similar abnormality in birth has been observed in cows and ewes in Australia[1~3,17] and in cows, ewes, and goats in Israel.[14] In Japan, no ovine or caprine cases of spontaneous infection with

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Akabane virus have been reported as yet. This condition may have been brought about, since the breeding season extends from autumn to winter for sheep and goats in Japan and differs from the period of activity of vectors transmitting Akabane virus. Parsonson et al.[16] and Hashiguchi et al.[4] carried out detailed studies on the fetopathogenicity of this virus for pregnant ewes. It was reported[9] that ewes were more susceptible to this virus than cows and that the virus had fetopathogenicity for pregnant goats.

Akabane disease live virus vaccine was developed by Kurogi et al. for the prevention of abnormality in birth in cows. It was proved to be used for calves and pregnant cows with safety.[11]

The present investigation was performed to determine whether this vaccine was available for the prevention of abnormality in birth in pregnant ewes, which have been considered to be highly susceptible to Akabane virus. In it, pregnant ewes were inoculated with the vaccine to examine its safety and immunogenicity. The results obtained are reported in this paper.

MATERIALS AND METHODS

Vaccine: The vaccine used was Akabane disease live virus vaccine (the TS–C2 strain) produced tentatively by the Laboratory of Viral Products, Biological Products Research Division, National Institute of Animal Health.[11,12] Lots 2 and 5 of this vaccine were supplied by Dr. Y. Inaba for the experiment. The infective titer was $10^{4.5}$ and $10^{5.3}$ $TCID_{50}$/ml for lots 2 and 5, respectively.

Virus: As high virulent virus for challenge inoculation, the OBE–1 strain[7] was used. It had been isolated from the brain of a bovine fetus and was in the form of freeze–dried product of emulsion of the mouse brain at the first passage level. Its infective titer was $10^{4.5}$ $TCID_{50}$/ml. The neutralization test was conducted with virus of the OBE–1 strain at the third passage level in HmLu–1 cell culture incubated at 37°C.

Outline of experiments: Twelve Corriedale ewes negative for antibody at 30～37 days of pregnancy were divided equally into two groups, group A for the potency test and group B for the safety test. Each ewe of group A was inoculated subcutaneously with 1 ml of lot 2 and each ewe of group B with 2 ml of lot 5. Both groups were examined for the appearance of viremia for 7 days after inoculation. The six ewes of group A and four ewes (at 45～53 days of pregnancy) of a non-immune control group called C were challenged by intravenous inoculation with 1 ml of the OBE–1 strain of high virulent virus 42 days after inoculation with the vaccine. They were observed for the appearance of viremia for 7 days and sacrificed 14 days after challenge inoculation. Fetuses were collected from them and their organs used for the recovery of virus. The six ewes of group B were allowed to give spontaneous birth to young, which were examined for abnormality and antibody of precolostral serum (PCS).

As materials for virus isolation, blood samples were collected daily from the ewes of groups A and B up to 7 days after inoculation with vaccine. Blood samples were also harvested daily from the ewes of groups A and C up to 7 days after challenge inoculation. Heparin (10 IU/ml) was added to all the samples, which were stored at −80°C until use. Various organs, femoral muscle, umbilical cord, and placenta were collected from each fetus and made to 10% emulsions in Earle's fluid containing 5% bovine serum. The emulsions were centrifuged at 1,000 rpm for 5 minutes. The resulting supernatants were used as inocula. These materials were stored at −80°C until use.

Serum samples were collected from group A before inoculation, at a week's intervals for 6 weeks after inoculation, and at the time of sacrifice, and from group B before inoculation, at a week's intervals for 4 weeks after inoculation, and at two weeks' intervals later up to the time of parturition. Such samples were collected from group C before and one week after challenge inoculation and at the time of sacrifice. Fetal serum (FS)

samples were harvested from the blood of the umbilical cord, and PCS samples from the blood of lambs before nursing with colostrum. All the samples were inactivated at 56°C for 30 minutes and stored at −20°C until use.

Cell culture: HmLu-1 cells originated from the lung of an infant hamster were obtained from a monolayer culture in a test tube (10×110 mm). The growth medium (GM) used was Eagle's minimum essential medium (MEM) to which had been added 10% bovine serum negative for antibody, antibiotics [100 U/ml of penicillin, 100 μg/ml of streptomycin, and 2 μg/ml of Fungizone (Squibb)], and 1.5% of 7.5% sodium bicarbonate. The maintenance medium (MM) used was Eagle's MEM to which had been added 5% bovine serum, antibiotics, and 2% of 7.5% sodium bicarbonate.

Recovery and titration of virus: The cell culture was washed once with Earle's solution. Then 0.1-ml amounts of each material were inoculated into ten tubes of cell culture and adsorption was allowed to take place at 37°C for 2 hours. After that, the cell culture was washed twice with Earle's solution. After addition of 0.5 ml of MM, it was incubated at 37°C in a rotating condition. When the cell culture inoculated with the initial material was negative for cytopathic effect (CPE), that in ten test tubes was pooled and subjected to two passages before results were read. In the estimation of virus infective titer, the inoculum was diluted tenfold serially with GM. Four tubes of cell culture were inoculated with 0.1 ml of each dilution to calculate the titer in $TCID_{50}$/g or ml.

Neutralization test: In the estimation of the neutralizing antibody titer of serum, the serum was diluted twofold serially with GM. Each series of dilution was mixed with an equal amount of 200 $TCID_{50}$/0.1 ml of virus suspension and incubated at 37°C for 1 hour. Then, 0.1 ml of each mixture was inoculated into two tubes of cell culture. The neutralizing antibody titer was expressed with the reciprocal of the highest serum dilution that inhibited CPE in more than one tube.

RESULTS

Clinical responses of pregnant ewes

Pyrexia or any other clinical symptom was manifested by no pregnant ewes after inoculation with vaccine or challenge inocu-

Table 1. *Viremia of pregnant ewes inoculated with vaccine*

Group	Pregnant ewe No.	Vaccination*1	Viremia							
			Days after vaccination							
			0	1	2	3	4	5	6	7
A	1	One dose of 1 ml each of Lot 2 vaccine	−*2	−	−	−	1.1*3	−	1.4	−
	2		−	−	−	−	−	−	−	−
	3		−	−	−	−	−	−	−	−
	4		−	−	−	−	−	−	−	−
	5		−	−	−	−	−	−	−	−
	6		−	−	−	−	−	−	−	−
B	7	One dose of 2 ml each of Lot 5 vaccine	−	−	−	−	−	−	−	−
	8		−	−	−	−	−	−	−	−
	9		−	−	−	−	−	−	−	−
	10		−	−	1.4	−	1.8	1.5	1.2	−
	11		−	−	−	−	−	−	−	−
	12		−	1.6	−	−	−	−	−	−

*1 Pregnant ewes were inoculated subcutaneously with vaccine.

*2 Virus was not recovered.

*3 $TCID_{50}$/ml (log)

lation with the high virulent OBE-1 strain.

Viremia of pregnant ewes inoculated with vaccine

An attempt was made to recover virus from the blood of the pregnant ewes inoculated with vaccine. The results obtained are shown in Table 1. Virus was recovered from one ewe (No. 1) of group A and two ewes

Table 2. *Antibody response of pregnant ewes inoculated subcutaneously with vaccine*

Group	Pregnant ewe No.	Vaccination*1	Neutralizing antibody titer Weeks after vaccination										
			0	1	2	3	4	5	6	8	10	12	14
A	1	One dose of 1 ml each of Lot 2 vaccine	<1	1	2	16	4	4	2				
	2		<1	1	2	16	16	4	16				
	3		<1	<1	<1	2	2	4	2				
	4		<1	1	1	32	32	4	8				
	5		<1	1	4	4	4	8	8				
	6		<1	2	8	8	2	4	4				
B	7	One dose of 2 ml each of Lot 5 vaccine	<1	1	8	32	16	NT*2	1	1	1	1	1
	8		<1	1	64	32	32	NT	2	2	1	1	2
	9		<1	1	4	2	1	NT	1	1	1	1	1
	10		<1	<1	64	128	32	NT	4	4	2	2	1
	11		<1	<1	8	32	4	NT	2	1	1	2	1
	12		<1	<1	8	32	32	NT	2	2	2	4	1

*1 See the footnote of Table 1.

*2 Not tested

Table 3. *Resistance of vaccinated pregnant ewes to challenge with virulent virus*

Group	Pregnant ewe No.	Vaccine inoculation*1			Challenge inoculation*2		
		Days pregnancy when inoculated	Demonstration of viremia (days after inoculation)	Neutralizing antibody titer at prechallenge inoculation	Days of pregnancy when challenged	Demonstration of viremia (days after inoculation)	Neutralizing antibody titer at slaughter (14 days after inoculation)
Vaccinated (A)	1	37	+(4, 6)	2	79	−	16
	2	36	−	16	78	−	64
	3	35	−	2	77	−	32
	4	37	−	8	79	−	128
	5	36	−	4	78	−	32
	6	34	−	4	76	−	64
Control(C) (Unvaccinated)	13			<1	45	+(3, 4, 5)	2
	14			<1	45	+(2, 3, 4)	4
	15			<1	52	+(3)	4
	16			<1	53	+(3)	8

*1 Pregnant ewes (Nos 1 to 6) were inoculated subcutaneously with 1 dose of 1 ml each of Lot 2 vaccine ($10^{4.5}$ $TCID_{50}$/ml).

*2 The vaccinated and unvaccinated control ewes were challenge-exposed intravenously with 1 ml of virulent virus (OBE-1 strain, $10^{4.5}$ $TCID_{50}$/ml) 42 days after vaccination.

Table 4. *Recovery of virus from fetuses obtained from vaccinated and challenged ewes*

Group*1	Fetus No.*2	Neutralizing antibody titer in fetal serum	Infective titer of fetal tissue and fluid: placenta	Amniotic fluid	Allantoic fluid	Umbilical cord	Brain	Spinal cord	Spleen	Liver	Kidney	Lung	Heart	Stomach	Intestine	Femoral muscle	Fetal blood
A	1	1	—*3	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	2	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	3	4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	4	<1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	5-A (Twin)	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	5-B (Twin)	4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	6	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
C	13	<1	3.8*4	—	—	—	4.5	2.4	—	—	2.3	2.8	2.3	—	—	—	—
	14	<1	2.5	—	—	—	2.5	—	—	—	—	—	—	—	—	—	—
	15	<1	3.5	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	16	<1	2.5	—	—	—	2.5	—	—	—	—	—	—	—	—	—	—

*1 The same group as indicated Table 3.

*2 The fetuses shown in this table bears the same numbers as its mothers shown in Table 3.

*3 Virus was not recovered.

*4 $TCID_{50}$/ml or g. (log)

(Nos. 10 and 12) of group B. Viremia was detected 4 and 6 days after inoculation in ewe 1, 2, 4, 5, and 6 days in ewe 10, and 1 day in ewe 12. The infective titer of virus in the blood was $10^{1.1}$~$10^{1.8}$ $TCID_{50}$/ml.

Antibody response of pregnant ewes inoculated with vaccine

Table 2 shows the rise and fall in neutralizing antibody titer of serum of pregnant ewes inoculated subcutaneously with vaccine. Of the 12 ewes inoculated, eight became positive for antibody 1 week after inoculation, exhibiting a titer of 1~2. All those ewes were positive 3 weeks after inoculation, exhibiting a titer of 2~128. The titer was 2~8 in six ewes at the time of parturition or at the time of sacrifice, or at 144 days of pregnancy.

Viremia of immunized and unimmunized pregnant ewes challenge exposed with virulent OBE-1 strain

Six ewes of group A and four ewes of group C were challenged by intravenous inoculation with the high virulent OBE-1 strain 42 days after inoculation of the six ewes with vaccine. The results obtained are indicated in Table 3. When challenged with the OBE-1 strain, the ewes of group A were at 76~79 days of pregnancy and presented a serum antibody titer of 2~16, and those of group C at 45~53 days of pregnancy and a serum antibody titer of <1. As a result, those of group A were all negative for viremia, and virus was recovered from the blood of all the ewes of group C 2~5 days after challenge inoculation. The infective titer of virus was $10^{1.1}$~$10^{1.7}$ $TCID_{50}$/ml.

Recovery of virus from fetuses and neutralizing antibody in fetal sera

Six ewes of group A and four ewes of group C were sacrificed 14 days after challenge with the high virulent virus. An attempt was made to recover virus from fetal organs collected from them. Table 4 shows the results obtained. The serum antibody titer was 16~128 in the ewes of group A and 2~8 in those of group C at the time of sacrifice. Seven fetuses were collected from

Table 5. *Responses of pregnant ewes, and their fetuses and newborn lambs to vaccine*

Pregnant ewe No.	Ewes				Fetuses and lambs		
	Days of pregnancy when inoculated*1	Demonstration of viremia (days after inoculation)	Duration of pregnancy (days)	Neutralizing antibody titer in sera at delivery or slaughter	Lamb or fetus No.	Gross finding	Neutralizing antibody titer in precolostral serum
7	30	—	169	4	7	Normal	2
8	36	—	151	2	8	Normal	4
9	36	—	152	4	9	Normal	4
10	33	—	150	2	10	Normal	2
11*2	37	+ (2, 4, 5, 6)	Sacrificed on 144 days	8	11-A (Twin) 11-B	Normal Normal	2 2
12		+ (1)	sterility				

*1 Pregnant ewes were inoculated subcutaneously with 2 ml of vaccine (Lot. 5, $10^{5.3}$ $TCID_{50}$/ml).

*2 This ewe was sacrificed due to mastitis.

the ewes of group A. No virus was recovered from the placenta or any organ from them. Virus was recovered from the brain of three of four fetuses harvested from group C, the virus infective titer being $10^{2.5}\sim10^{4.5}$ $TCID_{50}$/g. It was recovered also from the spinal cord, lung, heart, and stomach of ewe 13, the virus infective titer being $10^{2.3}\sim10^{2.8}$ $TCID_{50}$/g. It was recovered from the placenta of all the ewes inoculated, the virus infective titer being $10^{2.5}\sim10^{3.8}$ $TCID_{50}$/g.

The four fetuses harvested from group C were negative for antibody in FS. Of the seven fetuses harvested from group A, six were positive for this antibody, showing a titer of 1∼4.

Gross finding and neutralizing antibody in precolostral sera of lambs from ewes inoculated with vaccine

Antibody which seemed to have been produced by inoculation with vaccine was observed in FS of group A. Therefore, the pregnant ewes of group B were inoculated with vaccine alone and allowed to give spontaneous birth to young. Then, newborn lambs were examined for general conditions and antibody in PCS. The results obtained are indicated in Table 5. Four ewes presented normal parturition after a gestation period of 150∼169 days. The lambs born from them were free from abnormality in the body form and any dysfunction. Their brain was macroscopically normal. Ewe 11 was suffering from mastitis and sacrificed at 144 days of pregnancy. She harbored twin fetuses showing normal development. Ewe 12 was sterile.

The serum antibody titer was 2∼8 in the pregnant ewes at the time of parturition or sacrifice. Antibody was found in FS of two ewes and PCS of four ewes, being 2∼4 in titer. Accordingly, it was clarified that there was a fetal infection with vaccine virus.

DISCUSSION

Kurogi et al.[11] carried out detailed studies on the pathogenicity and immunogenicity of the TS-C2 strain of low virulent virus for cattle. According to them, this strain caused no pyrexia, leukopenia, or viremia in calves or pregnant cows, regardless of the route of inoculation. No virus was recovered from the fetus harbored by any inoculated cow. No antibody was found either in FS or PCS. From these results, they[11] concluded that the TS-C2 strain was attenuated to such extent that it could not induce fetal infection. Furthermore, pregnant cows immunized with this strain produced effective neutralizing antibody and could protect fetuses from an attack by high virulent virus. Therefore, this strain has been recommended as a candidate

strain for the preparation of live virus vaccine for the prevention of Akabane disease in cattle. It has been reported, however, abnormal birth caused by Akabane virus occurs not only in cows but also in ewes and goats.[2,4,5,9,14,16] Naturally, it is desirable that Akanebe disease live virus vaccine will be prepared from an effective low virulent strain safe for these animals.

The present investigation was performed in Sapporo, Hokkaido, which has been free from spontaneous infection of cows and ewes with Akabane virus. For it, Akabane disease live virus vaccine was prepared tentatively from the TS-C2 strain and inoculated into ewes to examine safety and immunogenicity for these animals. As a result, there was no problem on the immunogenicity of this vaccine for ewes. The ewes presented better immune responses than cows. Their fetuses could be prevented from an attack by high virulent virus. Two problems, however, have been pointed out on the safety of this vaccine. One of them is that viremia was noticed in three (Nos. 1, 10, and 12) of pregnant ewes inoculated with vaccine. It is considered that Akabane virus is transmitted by arthropods in the field. If a pregnant ewe is inoculated with the vaccine, there will be a sheep-vector-sheep cycle in the transmission of the virus and the ewe may be a source of transmission. It is also suggested that a back mutation may possible take place.

The second problem is that new-born lambs derived by spontaneous delivery from ewes inoculated with vaccine alone and the twin fetuses collected from ewe 11 showed normal growth without any abnormality in body shape or in the macroscopical finding of the brain. Nevertheless, antibody was present in FS and CPS in all the six cases, revealing the occurrence of fetal infectoin clearly.

When studies were made on the pathogenicity of the OBE-1 strain, which is an original strain of the vaccine, for pregnant ewes, viremia was found in all the ewes inoculated and abnormal changes were noticed in 13 (56.5%) of 23 neonatal lambs born from ewes inoculated with the OBE-1 strain at 28~69 days of pregnancy.[4] These changes were ankylosis of the limbs, scoliosis, hydranencephaly, porencephaly, stillbirth with dwarfism, and death after birth with dwarfism and weakness. As compared with the vaccine prepared from the OBE-1 strain, that prepared from the TS-C2 strain can be considered to have been attenuated to a large extent.

In brief, this vaccine is assumed to be safe and effective for cows. It induces, however, viremia in ewes, forming a source of transmission of Akabane disease. Besides, it causes fetal infection at a very high frequency when inoculated into pregnant ewes. It gives rise to a possibility of producing abnormal lambs. Therefore, it is not adequate for this vaccine to be used for the prevention of Akabane disease in ewes even in the non-pregnant stage.

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