アカバネウイルスに分類される新分離株（イリキ株）に起因する子牛の非化膿性脳炎

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Encephalitis of Cattle Caused by Iriki Isolate, a New Strain Belonging to Akabane Virus

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ABCTRACT. A disease characterized by nervous signs was found in 10 calves in two districts in Kagoshima Prefecture, Japan, from October to November, 1984. Histopathological changes of nonpurulent encephalitis were found in every case. An agent, named Iriki isolate, was isolated from the cerebellum of a calf in HmLu-1 cell cultures. All of the affected calves possessed neutralizing antibody to the virus. A high seropositive rate to the virus in cohabiting cattle and cattle kept in the epizootic area, and seroconversion to the virus in 1984, were disclosed. Experimental infection of calves with Iriki isolate produced severe nervous signs and histopathological changes similar to those of the natural infection. These seroepidemiological findings and animal experiments established that Iriki isolate is the causative agent of the disease. Iriki isolate was considered as a variant of Akabane virus since the virus showed cross reaction with Akabane virus in virus neutralization tests.—KEY WORDS: Akabane virus, cattle, encephalitis, Iriki isolate, Simbu serogroup.

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It is well known that encephalitis of cattle is caused by infection with Japanese encephalitis virus, malignant catarrhal fever virus, pseudorabies virus and rabies virus. Although cases of the encephalitis caused by these viruses are rare in Japan, cases of encephalitis due to unknown reason are present in no small numbers.

In 1984, a disease characterized by nervous signs occurred among calves in Kagoshima Prefecture, Japan. This paper describes clinical, histopathological and virological studies of the disease.

MATERIALS AND METHODS

Etiological examination. For the isolation of virus from affected calves, the supernatant of a 20% homogenate of each tissue shown in Table 1 was inoculated into 4 tube cultures of HmLu-1 cells. After incubation in a roller drum at 37°C for 7 days, each culture fluid was subinoculated into freshly prepared cell cultures. This subinoculation process was repeated twice. Isolation of a virus was determined by the appearance of a cytopathic effect in cultures after the 2nd subinoculation.

The tissues used for virus isolation were examined for bacteria. Each tissue was incubated on a blood agar plate and DHL agar plate under aerobic conditions and on a GAM agar plate under anaerobic conditions at 37°C.

Serum samples. Sera of affected calves were collected on the day of sacrifice. Serum samples of cattle cohabiting with affected calves were collected in May and July 1985, and those of cattle kept in the epizootic area were collected in November and December in 1984. Sera collected from July to November 1984 from sentinel calves (about 6 months old at the beginning of the test) which had been kept in the area were also used.

Virus neutralization test. This test was

Table 1. Isolation of virus from calves with nervous signs

Specimen	Calf No.								
	1 (10)[a]	2 (4)	3 (3)	4 (16)	5 (0.5)	6 (3)	7 (3 days)	8 (4)	9 (15)
Cerebrum	–[b]	–	–	–	–	–	–	–	–
Cerebellum	ND[c]	ND	ND	ND	+	ND	ND	ND	ND
Spinal cord	ND	–	ND	–	–	–	–	–	ND
Lung	–	–	–	+	–	–	–	–	–
Liver	–	–	–	+	–	–	–	–	–
Spleen	–	–	–	+	–	–	–	–	–
Tonsil	–	–	–	+	–	–	–	–	–
Lymph nodes[d]	–	–	–	+	–	–	–	–	–
Blood	–	–	–	+	–	–	–	–	–

a) Numbers in parentheses show age of calves in months, except No. 7 which is shown in days.
b) Positive and negative for virus isolation.
c) Not done.
d) Mesenteric lymph nodes.

performed by the constant virus–diluted serum method [8] and the diluted virus-constant serum method. The former was carried out in microplates and used mainly for serological surveys. The antibody titer was expressed as the reciprocal of the highest serum dilution which inhibited cytopathic effect of indicator virus. In the latter test, sera to be tested were diluted 1:10 or 1:20 in a maintenance medium and heated at 56°C for 30 minutes. Then each serum or a maintenance medium which was used as a control was mixed with an equal volume of one of the serial tenfold virus dilutions. After being held at 37°C for 1 hour, 0.1 m*l* of each mixture was inoculated into each of 4 tube cultures. Infectivity of each mixture was determined by the appearance of a cytopathic effect after 7 days of incubation at 37°C and expressed in terms of $TCID_{50}$ per m*l*. The neutralizing antibody titer is expressed as the log-index (neutralizing index) being the difference in the logarithm of the virus titers between the test serum and the control.

Viruses. A new agent, named Iriki isolate (strain 526CE) was used at the 7th passage level in HmLu-1 cell cultures after the isolation. Three strains of Akabane virus (JaGAr 39, OBE-1 and NBE-9) and Aino (strain JaNAr 28), Douglas (strain CSIRO 150), Peaton (strain CSIRO 110) and Tinaroo (strain CSIRO 153) viruses [4, 9] were also used.

Cell cultures. HmLu-1 cell cultures were used for all virological and serological studies. Details of the method were described previously [6].

Antisera. One calf each was inoculated intravenously with one of the 3 strains of Akabane virus or Iriki isolate (the 526CE strain). Serum collected 5 weeks after inoculation was used as antiserum against the respective viruses. Rabbit antisera to the JaGAr 39 strain (prototype strain) of Akabane virus and Iriki isolate (the 426CE strain) were prepared by the method described previously [13].

Electronmicroscopy. HmLu-1 cells infected with Iriki isolate were observed with an electronmicroscope after routine treatment [1].

Experimental infection. A 2-day-old colostrum-deprived calf (Holstein), No. 958,

and a 6-month-old calf (Holstein), No. 957, which had no antibody to Iriki isolate were inoculated intracerebrally with 2 m*l* of the supernatant of a 10% emulsion of the brain of suckling mice infected with Iriki isolate. The history of the virus was 7 passages in HmLu-1 cell cultures, 1 passage in a calf and 2 passages in suckling mouse brain. The infectivity of the inoculum was $10^{5.3}$ and $10^{6.2}$ $TCID_{50}$/m*l* for No. 957 and No. 958, respectively.

RESULTS

Clinical and epidemiological features of the natural infection. Ten calves with signs characteristic of encephalitis were observed in 10 farms in 2 districts (Hokusatsu and Nansatsu) in Kagoshima Prefecture, the southernmost prefecture of the main Lands of Japan, from late October to the end of November, 1984. These farms kept a few to 150 head of cattle. The calves were from 3 days to 16 months old (Table 1). Six and 4 of the calves were Japanese Black and Holstein breeds, respectively. Five of the calves were males and 5 were females.

One of the 10 calves died of the disease 5 days after the onset with marked nervous signs. The remaining 9 calves were sacrificed when they showed severe signs and prognosis was judged to be very poor. The duration from the onset to sacrifice was 2 to 3 days in 5 cases and 1, 6, 12 and 16 days in 1 case each. The first signs observed were lassitude and anorexia, which were followed by foamy salivation and marked nervous signs such as tremor, stretching of the legs and ataxia-like circling and collision with a barrier. Then it became difficult for them to stand. Rapid breathing and pupillary contraction were also observed in about half of the calves. Fever, higher than 40°C, was observed in only 2 cases.

Histopathological features of the natural infection. Hyperemia and malacia were observed in the brain. Hyperemia and edema in the lung, petechiae in the endocardium, hyperemia and petechiae in the rumen and small intestine, and whipworm infestation in the large intestine were observed in some cases.

Microscopically, severe perivascular infiltration of lymphocytes, glial cell proliferation, neuronal degeneration, and hemorrhage were observed in the central nervous system in all the cases, and they were particularly severe in the brain stem and spinal cord (Fig. 1-A and -B). Gitter cells were seen in some cases.

Etiological investigation. No significant bacterium was detected in the tissues examined. Virologically, one virus was isolated in the cerebellum of one calf, No. 5 (Table 1). Moreover, another virus was isolated in various tissues other than the brain and spinal cord of one calf, No. 4. However, because it has been disclosed that the latter virus, named Kawanabe virus, has no relationship with the disease, we have described the studies on that virus in a separate paper [10]. Accordingly, only the studies on the former virus, named Iriki isolate after the place of isolation, are described in this paper.

Morphogenesis of the virus. Electronmicroscopically, virus particles were seen outside the cytoplasmic membrane of normal-appearing cells. They were round, oval or elongated and their diameter varied from 90 to 100 nm. A membrane-like envelope was sometimes observed (Fig. 2). These morphological features were very similar to those of Akabane virus [1].

Serological relationship between Iriki isolate and Simbu serogroup viruses. Since the morphological observation revealed that the Iriki isolate has many similarities to Akabane virus, a serological comparison of Iriki isolate with Akabane, Aino, Douglas, Peaton and Tinaroo viruses, which belong to the Simbu serogroup of genus *Bunyavirus*

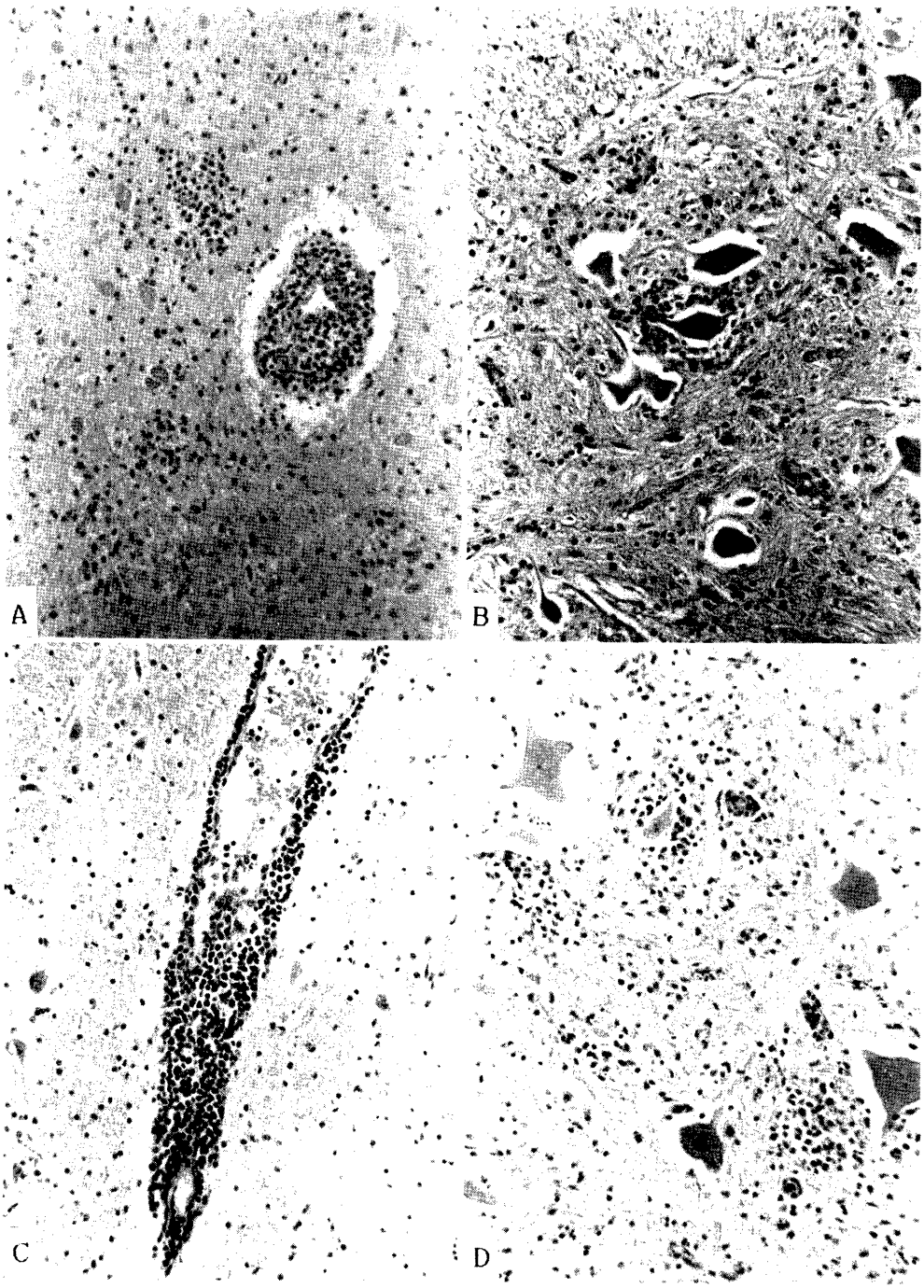

Fig. 1. Histopathological features of naturally infected calves (Nos. 5 and 8) and an experimentally infected calf (No. 985). HE staining, ×125. A. Medulla oblongata of No. 5. Severe perivascular infiltration of lymphocytes and plasma cells and glial nodules are seen. B. Ventral horn of the spinal cord of No. 8. Glial cells proliferate densely around the neuron and diffusely in the gray matter. C. Medulla oblongata of No. 985. Lymphocytes infiltrate perivascularly. D. Ventral horn of the spinal cord of No. 985. Glial cells proliferate around the neuron.

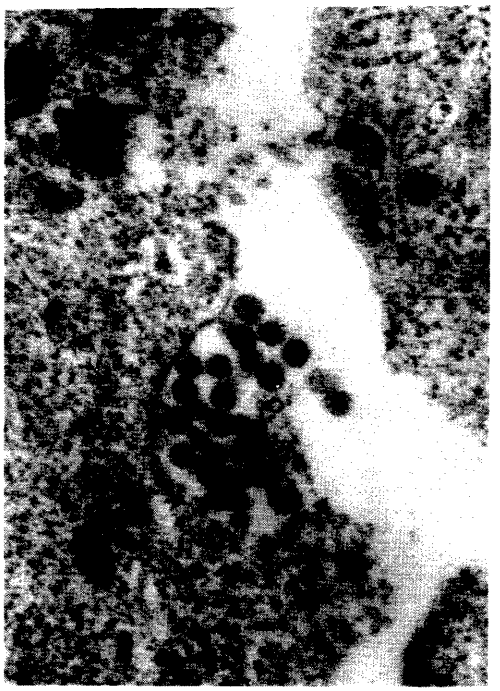

Fig. 2. Electronmicroscope of HmLu-1 cells inoculated with Iriki isolate. Virus particles are seen near the normal-appearing cell. ×40,000.

Table 2. Neutralization test of viruses in Simbu serogroup with antiserum to Iriki isolate

Virus	Antiserum to Iriki isolate
Iriki	3.3[a)]
Akabane (OBE-1)	3.8
Aino	−0.3
Douglas	−0.2
Peaton	1.3
Tinaroo	1.3

a) Neutralizing index.

and are known to be present in Japan [7] was performed. As shown in Table 2, Akabane virus was strongly neutralized and Peaton and Tinaroo viruses were weakly neutralized by the antiserum to Iriki isolate. A cross-neutralization test between Iriki isolate and Akabane virus was performed using sera collected sequentially from calves infected with Iriki isolate or Akabane virus. As shown in Table 3, calves infected with Iriki isolate developed antibody to Akabane virus as well as homologous virus. In contrast, calves infected with Akabane virus

Table 3. Development of neutralizing antibody in calves inoculated with Iriki isolate or Akabane virus against the both viruses

Calf No.	Virus inoculated	Weeks after inoculation	Neutralizing antibody titers against Iriki virus	Neutralizing antibody titers against Akabane virus
956	Iriki (526CE)	0	<4[a)]	<4
		4	4	4
		7	8	4
976	Iriki (526CE)	0	<4	<4
		4	4	4
799	Akabane (JaGAr39)	0	<4	<4
		4	<4	64
		7	<4	256
800	Akabane (JaGAr39)	0	<4	<4
		4	<4	4
		7	<4	4

a) Reciprocal of the highest serum dilution that inhibited a cytopathic effect.

Table 4. Antigenic relationship between Iriki isolate and Akabane viruses

Virus (strain)	Antiserum			
	Iriki isolate	Akabane virus		
	(526CE)	(JaGAr39)	(OBE-1)	(NBE-9)
Iriki (526CE)	3.3[a)]	1.3	2.7	0.8
Akabane (OBE-1)	3.8	4.6	4.9	6.0

a) Neutralizing index.

produced antibody against only homologous virus. A further comparison between the 2 viruses was performed using antisera against 3 strains of Akabane virus (Table 4). Iriki isolate was strongly neutralized by homologous antiserum and weakly at different degrees by the 3 antisera against Akabane virus. The 2 viruses were also compared by using rabbit antisera against Iriki isolate and the prototype strain (JaGAr 39) of Akabane virus (Table 5). Similar to the previous test, Iriki isolate showed very weak heterologous reactivity, while Akabane virus demonstrated strong heterologous as well as homologous reactivity.

Table 5. Cross-neutralization test between Iriki isolate and Akabane virus

Virus (strain)	Immune serum	
	Iriki isolate (526CE)	Akabane virus (JaGAr39)
Iriki (526CE)	128[a)]	4
Akabane (JaGAr39)	128	64

a) Reciprocal of the highest serum dilution that inhibited a cytopathic effect.

Seroepidemiological surveys. All calves that died of or suffered from the disease had antibody titers of 4 to 32 against Iriki isolate and those of 4 to 64 against Akabane virus (Table 6). Sixty five (63.7%) of 102 cattle which cohabited with the affected calves possessed neutralizing antibody titers of 2 to 32 to the Iriki isolate, and 46 (33.6%) of 137 cattle which were kept in the same area also possessed the antibody. Most of the positive sera also reacted with Akabane virus. Some of antibody-negative sera against Iriki iso-

Table 6. Neutralizing antibody titers of serum collected from calves with nervous signs

virus used (strain)	Calf No.[a)]									
	1	2	3	4	5	6	7	8	9	10
Iriki (526CE)	4	16	16	8	32	32	8	4	16	32
Akabane (JaGAr39)	4	8	32	16	8	8	16	16	8	64

a) Details of the calves are shown in Table 1. Calf No. 10 (6 months old) was tested after death.

Table 7. Clinical and virological findings in calves inoculated intracerebrally with Iriki isolate

Calf No.	Items tested	Days after virus inoculation											
		0	1	2	3	4	5	6	7	8	9	10	11
957	General signs[b]	−	−	−	−	+	+	+	++	+++	++++		
	Nervous signs[c]	−	−	−	−	−	−	−	−	±	++++		
	Viremia	−	−	1.5[d]	2.0	2.7	2.3	2.7	−	−	−		
958	General signs	−	−	−	−	−	−	−	+	+	+	++++	++++
	Nervous signs	−	−	−	−	−	−	−	−	−	−	±	++++
	Viremia	−	−	0.7	2.0	2.7	3.5	2.8	−	−	−	−	−

a) The 2 calves showed no apparent febrile response and no leukopenia.
b) −: no signs, + ~ ++: anorexia and lassitude, +++ ~ ++++: staggering and inability to stand.
c) − ~ ++++: degree of nervous sings.
d) log $TCID_{50}$/m*l*. −: not detected.

late reacted with Akabane virus. Prevalence of the virus was tested for by using the sera collected monthly from sentinel calves in the epizootic area in 1984. Seroconversion to Iriki isolate as well as Akabane virus was detected in November in 5 of 20 sentinel calves.

Experimintal disease. Two calves inoculated intracerebrally with the isolate showed no apparent febrile responses and no leukopenia during the observation period. They had loss of appetite and vigor from the 4th and 7th day, respectively, and showed staggering thereafter. Their general condition became progressively worse and they were finally unable to stand on the 9th and 11th day. On that day, they suddenly developed marked nervous signs such as tremor, nystagmus, opisthotonus and stiffness of the legs, and secreted a large amount of saliva. Then they showed a rapid drop in body temperature, became moribund, and were sacrificed (Table 7). The virus was detected from the 2nd day to the 6th day in the plasma of both calves. Neutralizing antibody was not detected in either calf at the time of sacrifice.

Recovery of the virus from tissues of the 2 calves is shown in Table 8. The virus was detected in the brain and spinal cord of both calves and in the liver and mesenteric lymph nodes of one.

Table 8. Distribution of virus in calves inoculated intracerebrally with Iriki isolate

Specimen	Calf No.	
	957	958
Cerebrum	1.8[a]	3.0
Cerebellum	2.5	3.2
Medulla oblongata	ND[b]	3.5
Spinal cord	3.5	2.7
Lung	—[c]	—
Liver	1.7	—
Spleen	—	—
Kidney	—	—
Mesenteric lymph node	3.3	—
Blood	—	—

a) log $TCID_{50}$/g.
b) Not done.
c) Not detected.

Histopathologically, the principal lesions were observed in the central nervous system in both cases. Lymphocytes infiltrated perivascularly to form a few layers. Many glial nodules were observed in both white and gray matter. These lesions were severe in the midbrain, medulla oblongata, and spinal cord, and mild in the cerebrum and cerebellum. In the spinal cord, the lesions were more severe in the ventral horn than in the

dorsal horn. Neurons involved in the glial nodules were sometimes degenerated (Fig. 1-C and-D).

DISCUSSION

A disease characterized by marked nervous signs occurred in Kagoshima Prefecture among calves from October to November 1984. One virus, named Iriki isolate, was isolated from one of the affected calves. Antibody to Iriki isolate was found in all of the affected calves and also at a high ratio in cohabiting cattle. The rate of positive antibody to the virus was also relatively high in the epizootic area, but it was lower than that for the cattle cohabiting with the affected calves. Furthermore, seroconversion to the virus was observed in sentinel calves in the area in 1984 suggesting the prevalence of the virus. However, since Iriki isolate has serological cross reactivity with Akabane virus further investigations will be required to distinguish the infection with Iriki isolate from that with Akabane virus.

Experimental infection with Iriki isolate caused clinical signs similar to those of the natural infection and identical and characteristic histopathological changes such as severe perivascular infiltration of lymphocytes and glial nodules. These findings seem sufficient enough to identify the Iriki isolate as the causal agent of the epizootic encephalitis in Kagoshima Prefecture. The low rate of isolation of the virus from affected calves seems to be due to the presence of neutralizing antibody in the serum.

Iriki isolate showed a strong similarities to Akabane virus morphologically. Although Iriki isolate showed low cross reactiviy with antiserum to Akabane virus, Akabane virus reacted well with antiserum against Iriki isolate. Accordingly, Iriki isolate will be considered as a variant of Akabane virus, a member of the Simbu serogroup of genus *Bunyavirus*. It is known that Sabo, Shamonda, Tinaroo and Yaba-7 viruses react with antiserum to Akabane virus [2]. Therefore, it is necessary to investigate the serological relationship between Iriki isolate and those viruses in a future study.

There are many viruses belonging to the Simbu group which infect cattle. However, the pathogenicity of most of the viruses, except Akabane virus, is obscure [12]. As far as we can tell from our limited knowledge, no virus in this group causes encephalitis in nature , although some of them cause encephalitis upon experimental intracerebral inoculation [5, 11]. Infection with Akabane virus is reported every year in Japan, but no case of encephalitis caused by the virus has been reported. In exprimental intracerebral infection, Akabane virus caused only slight and transient nervous signs in 2 of 6 calves [5]. Therefore, Iriki isolate is particularly interesting as a new agent which has higher virulence than conventional Akabane virus and has the ability to cause encephalitis by peripheral infection.

One of the affected calves was 3 days old (Table 1). This fact indicates transplacental infection of the virus to the fetus. Thus, it is likely that Iriki isolate causes abortion, premature birth, stillbirth, and congenital abnormalities in cattle, as does Akabane virus [3, 4]. Since antibody to Akabane virus has only slight ability to neutralize Iriki isolate, as shown in this study, a vaccine against Akabane disease may be ineffective against Iriki isolate infection. This presumption suggests the importance of developing a vaccine against the virus.

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要　　約

アカバネウイルスに分類される新分離株，イリキ株，に起因する子牛の非化膿性脳炎：宮里俊光・三浦康男[1)]・長谷　学・久保正法[1)]・後藤義之[1)]・甲野雄次[1)]（鹿児島県中央家畜保健衛生所，[1)]家畜衛生試験場九州支場）——1984年10月から11月にかけて鹿児島県下で神経症状を示す疾病が10頭の子牛に発生した．そのうち1頭の小脳から HmLu-1 細胞に CPE を示すウイルスが分離され，イリキ株と名付けられた．発症牛は全例に，また同居牛も高率に本ウイルスに対する抗体を保有していた．おとり牛を用いた調査により，1984年には本ウイルスに対する抗体の陽転がみられ，このウイルスの流行が示唆された．イリキ株は中和試験によりアカバネウイルスと片側交差を示すことから，アカバネウイルスに分類されると思われる．電子顕微鏡的にも本ウイルスはアカバネウイルスと類似していた．本ウイルスの実験感染の結果，子牛は自然感染例と類似の致死的な神経症状及び脳病変を示した．以上の結果から，イリキ株は今回報告された子牛の非化膿炎の原因であることが明らかとなった．