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LETTER

Characterization of a recombinant Akabane mutant virus with knockout of a nonstructural protein NSs in a pregnant goat model

Dear Editor,

Akabane virus (AKAV), an *orthobunyavirus*, is transmitted primarily by biting midges and is widely distributed throughout the world except the Europe. AKAV was first isolated from mosquitoes in Japan (Oya et al., 1961). Although pregnant cows, ewes, and goats infected with AKAV exhibit no clinical signs of disease, *in utero* infections result in abortion, premature birth, stillbirth, and congenital deformities such as arthrogryposis-hydranencephaly syndrome (Kurogi et al., 1976), causing economic losses in the livestock industry. The live, attenuated vaccine strain, TS-C2, was derived from the OBE-1 strain as a temperature sensitive mutant (Kurogi et al., 1979). Although vaccination has reduced the prevalence of the disease, antigenic and pathogenic variants of AKAV have been isolated (Lee et al., 2002; Ogawa et al., 2007a); for example, a variant Iriki strain was isolated from a calf with nonsuppurative encephalitis and neurological symptoms in Japan (Miyazato et al., 1989) and it shows low antigenic cross-reactivity with the reference strain in neutralization tests (Akashi and Inaba, 1997). Therefore, it is necessary to reconsider the vaccination strategy to effectively control the disease. Here we evaluated characters of a mutant virus with knockout of a nonstructural protein NSs, which acts as type I interferon antagonist and is involved in the regulation of host protein synthesis (Weber et al., 2002), by experimentally infecting pregnant goats. The pregnant goat model might be useful for AKAV studies, as suggested by previous reports of experimental transplacental infection of caprine fetuses (Kurogi et al., 1977).

The recombinant TS-C2 (rTTT) and its mutant virus with knockout of NSs (rTTTΔNSs) were generated by reverse genetics in our previous study (Takenaka-Uema et al., 2016). rTTTΔNSs grew at a slower rate than rTTT in cell culture, although it reached a maximal yield at 48 hours post infection, which was equivalent to those of rTTT (Takenaka-Uema et al., 2016). Seven pregnant and five non-pregnant Shiba goats (9 months to 30 months) were obtained from the Animal Resource Science Center, Graduate School of Agricultural and Life Sciences, University of Tokyo. After 3 days of acclimatization, two pregnant goats were inoculated with 3 mL (2.3–4.6 $\times$ 10^7PFU) of wild-type (wt) Iriki strain, rTTT, or TTTΔNSs intravenously via the cervical vein at 37 to 49 days gestation. After inoculation, body weights were measured, and rectal body temperatures and other general clinical variables were examined. Blood samples were collected post inoculation, and white blood cells (WBCs) were isolated. Fetuses were removed from the uteruses for autopsy at 3 weeks post inoculation (wpi). Since the design of this goat model is qualitative rather than quantitative and takes the principles of replacement, reduction, and refinement in animal experiments into account, the present study is limited by the number of animals that were used to assess the potentiality of a recombinant virus.

Clinical signs were absent in all inoculated goats during the experimental period. Sera obtained at the time of virus inoculation and at 1-week intervals over the next 3 weeks were analyzed for the presence of virus-neutralizing (VN) antibodies (Ogawa et al., 2007a). Five of the six goats inoculated with virus had no antibodies against the virus at the time of inoculation, whereas one goat (#3) had a detectable antibody titer (8) due to unknown reasons, and the highest antibody titer (181) elicited in this goat was at 2 wpi (Table 1), possibly because of immune boosting. All inoculated goats developed detectable VN antibodies at 1 wpi, and their antibody levels peaked at 2 or 3 wpi. For comparison, we evaluated the antibody responses to the TS-C2 strain-based commercial vaccine (Nisseiken, Oume, Japan) in goats; the highest antibody titers (15.0 ± 6.9) were observed at 2 wpi. These data suggest that the rTTTΔNSs strain elicits an immune response in goats equivalent to that of the current vaccine virus.

At autopsy, the cerebrum, cerebellum, brain stem, spinal cord, visceral organs, and placenta were collected from the fetuses at 3 wpi. Samples were fixed in 10% neutral buffered formalin and processed for paraffin embedding. Serial sections of each tissue mounted on slides were stained with hematoxylin-eosin for histopathologic-

Table 1. Virus-neutralizing antibody titers in pregnant goats inoculated with wt Iriki or recombinant AKAV

Virus	Pregnant goat	Weeks post inoculation			
		0	1	2	3
Mock (PBS)	# 1	< 2	< 2	< 2	< 2
wt Iriki	# 2	< 2	7	45	45
	# 3	8	23	181	108
rTTT	# 4	< 2	2	27	14
	# 5	< 2	7	27	19
rTTTΔNSs	# 6	< 2	7	16	23
	# 7	< 2	16	54	32
Vaccine TS-C2[a]	5 non-pregnant goats	< 2	< 2	4–23	5–16

Notes: [a:] For comparison, a commercial vaccine (TS-C2 strain) was inoculated into non-pregnant goats according to the manufacturer's instructions.

al examination and immunostained to detect AKAV antigen using the Envision polymer method (Dako Envision system, Agilent Technologies, Tokyo, Japan). Rabbit antiserum against the OBE-1 strain (Tsuda et al., 2004) was used as the primary antibody. Immunoreactivity was visualized with 3, 3'-diaminobenzidine. Fetuses removed from the dams inoculated with wt Iriki exhibited gross changes (Figures 1A-a, b). One fetus (#2-1) removed from dam #2 had hydranencephaly characterized by thinning of the cerebral hemispheres and enlargement of the lateral ventricles (Figure 1A-d). The brain of another fetus (#2-2) from the same litter was grossly normal, and no viral antigens were detected. The cerebrum of a fetus (#3-1) removed from dam #3 was pale and thin in appearance (Figure 1A-b) and presented enlargement of the lateral ventricle (Figure 1A-c). No fetuses from dams challenged with rTTT or rTTTΔNS or the uninfected control animal had brain changes that were detectable by gross observation (Figures 1A-b, c). No distinct encephalitis was observed in assessed tissues, even in fetal brains that showed gross abnormality by histopathological examination. AKAV antigens were detected in the cerebrum and brain stem of fetuses #2-1 and #3-1 from goats inoculated with wt Iriki. Viral antigens were widely distributed in the neuronal cells of the cerebral hemisphere, midbrain, and diencephalon (Figure 1A-e). No viral antigens were detected in fetal tissues from goats (#4–#7) that were inoculated with recombinant viruses.

WBCs from dams and the brain tissues of fetuses were examined for the presence of viral RNA by quantitative RT-PCR. Viral RNA was extracted from WBCs or fetal tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Two μL of RNA were used to synthesize cDNA using the PrimeScript RT Reagent Kit (Takara Bio, Shiga, Japan). For quantitative PCR, ak151f and ak231r primers (Stram et al., 2004) were used to amplify the AKAV cDNA, and the 18S rRNA primer pair (18S-qF and 18S-qR; sequences are provided upon request) was used to amplify an endogenous control gene. The reactions contained 12.5 μL of Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 2 μL of RT reaction product, 10 μmol/L each primer, and water to a final volume of 25 μL. Reactions were performed under the following cycling conditions (95 °C for 10 min, and 45 cycles of 95 °C for 15 s and 60 °C for 30 s) in a TP800 Thermal Cycler Dice Real Time System (Takara Bio). Although a high level of viral RNA was detected in dam #2 at 3 days post infection (dpi) (Figure 1B-a), no viral RNA was detected in other dams at this time point. No viral RNAs were detected in any of the dams at the other time points (0, 7, 14 and 21 dpi). The brain of fetus #2-1 showed a significantly higher viral RNA level than the brains of other fetuses (#3-2, #4-2, and #5-2) (Figure 1B-b). Low levels of viral RNA were also detected in fetuses from rTTT-inoculated goats, indicating the existence of a small amount of virus, whereas RNA levels in fetuses from rTTTΔNSs-inoculated goats were equivalent to the background level detected in the fetus from the control animal. These data suggest a disconnection between viremia in dams and virus transmission to fetuses in this animal model.

We (Ogawa et al., 2007b) and others (Bridgen et al., 2001) demonstrated that NSs was responsible for orthobunyavirus pathogenicity. Thus, deletion of NSs from the TS-C2 strain was expected to result in a more attenuated phenotype. In fact, rTTTΔNSs RNA was not detected in WBCs from dams inoculated with this virus or in the brains of their fetuses, whereas rTTT RNA was detected in the brain of the fetuses, demonstrating that rTTTΔNSs was highly attenuated.

In this pregnant goat model, the Iriki strain induced

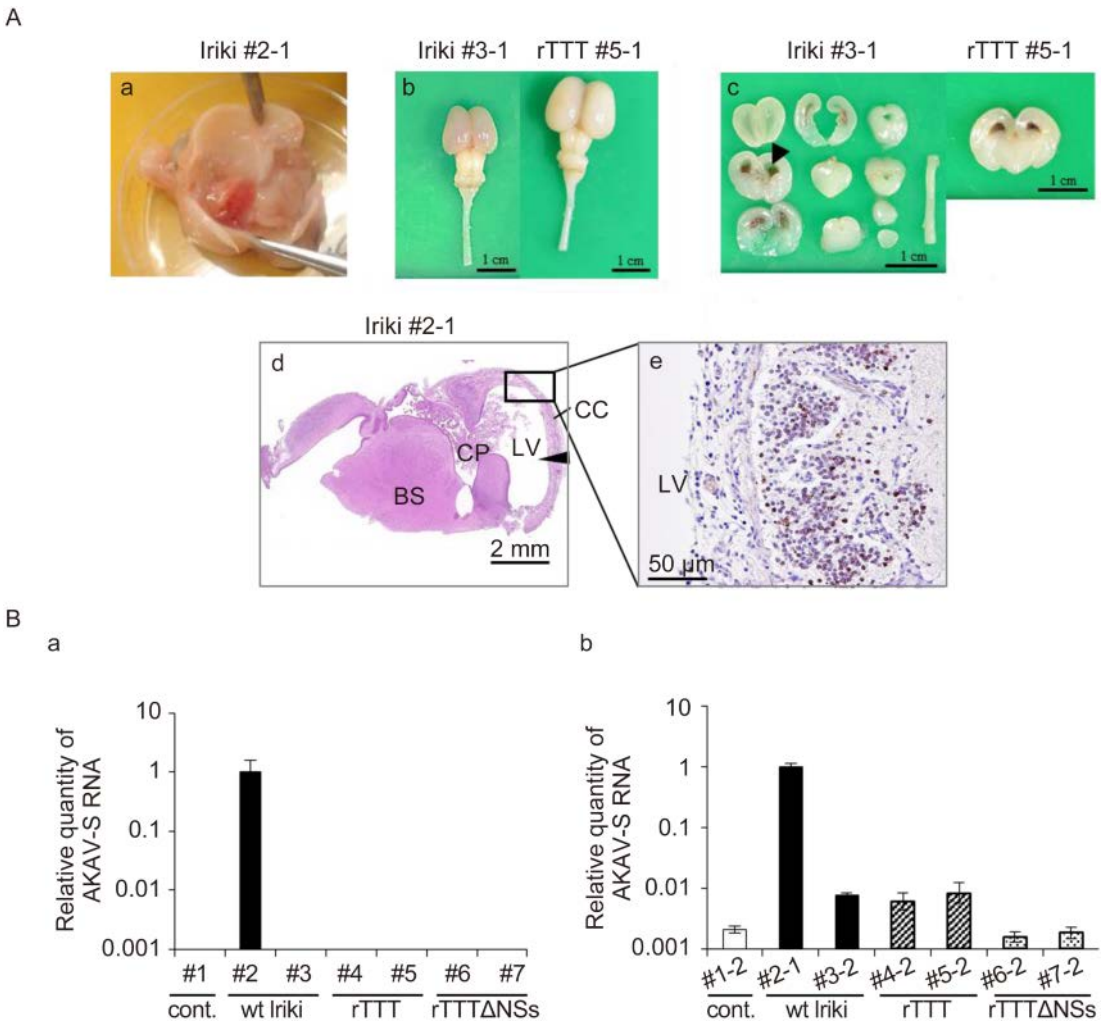

Figure 1. (A) Gross and histopathological findings in the fetal brains of the wt-Iriki-inoculated group. a: Fetal brain (#2-1) showing hydranencephaly. b: Pale and thin cerebrum of a fetal brain (#3-1) and a normal appearing brain (#5-1). c: Series of coronal sections of an altered fetal brain and spinal cord (#3-1) and normal sections (#5-1). The arrow indicates an enlarged lateral ventricle. d: Hematoxylin-eosin staining of a coronal section of fetal brain (#2-1). The arrowhead shows an enlargement of the cerebral ventricle. LV,lateral ventricle;CC,cerebrum cortex; CP,choroid plexus; BS,brainstem. e: Immunostaining of viral antigens. Higher magnification of the area within the box in panel (d). #2-1 shows abundant viral antigens in the parenchyma of the cerebral hemisphere. (B) a: Detection of viral RNA by quantitative PCR (qPCR) in the WBCs of pregnant goats inoculated with wt Iriki or recombinant virus at 3 dpi. b: Detection of viral RNA by qPCR in fetuses removed from inoculated pregnant goats. #1-2,#2-1,#3-2,#4-2,#5-2,#6-2,and #7-2 are fetuses from the #1,#2,#3,#4,#5,#6,and #7 dams shown in (a),respectively. The results shown are the mean ± SD of multiple measurements. Two replicates of qPCR showed equivalent results.

viremia in dams. The fetuses from these dams showed abnormalities such as hydranencephaly and hypoplasia of the cerebral hemispheres, and abundant viral RNA was detected in the brain. These results correspond with the pathology observed for natural transplacental infection of calves with AKAV (Konno and Nakagawa, 1982), which confirmed that the Iriki strain possesses not only neurovirulence but also teratogenicity. Thus, the pregnant goat model may be useful for the AKAV studies.

We showed that rTTTΔNSs has adequate humoral immunogenic response in goats. To demonstrate a vaccine potential of the rTTTΔNSs, we are planning to perform challenge experiments. This type of genetically manipulated virus could be used to produce antigenic-matching vaccine viruses to circulating AKAV strains. For example, vaccine candidates such as reassortants having the S and L segments of TS-C2 and M segment of the antigenic variants can be easily and robustly constructed

by using the reverse genetics system over conventional procedures for reassortant production by co-infection of two viruses. In addition, vaccines based on recombinant viruses lacking NSs, such as rTTTΔNSs, may allow for differentiation between infected and vaccinated animals (DIVA), if NSs antibodies can be consistently detected in animals infected with the wt virus, but not in vaccinated animals (McElroy et al., 2009). Evaluation of the potential for DIVA with an NSs-defective vaccine strain against AKAV infection is a future work.

FOOTNOTES

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