Chuzanウイルス不活化ワクチン開発のための基礎試験

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A Preliminary Study for Development of an Inactivated Chuzan Virus Vaccine

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ABSTRACT. A preliminary study for development of an inactivated vaccine for Chuzan virus was performed. The best propagation of the virus was observed in BHK21 cell cultures. The culture fluid after treatment with formalin had good immunogenicity in cattle and guinea pigs. Immunized calves did not develop leukopenia and viremia, which were observed in a control calf, after challenge inoculation.—KEY WORDS: Chuzan virus, immunity, inactivated vaccine.

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An epidemic of congenital abnormalities of calves with the hydranencephaly-cerebellar hypoplasia (HCH) syndrome occurred from autumn 1985 to spring 1986 in the Kyushu district in Japan [1, 2]. Chuzan virus, a new virus belonging to the Palyam subgroup of orbivirus [4], was strongly suggested as the causative agent of the disease by immunological and epidemiological investigations [1, 2, 4]. Furthermore, reproduction of the disease by experimental infection of pregnant cows with the virus was successful (Miura *et al.*, unpublished data).

Distribution of antibody for Chuzan virus in Japan was limited to the Kyushu and Okinawa districts and some areas in the Chugoku and Shikoku districts [2]. This distribution suggests the possibility of outbreaks of abnormalities with HCH syndrome in calves in wide spread districts in Japan in the future, and this possibility prompted us to develop a vaccine for the virus. In this paper we describe a preliminary study for development of such a vaccine.

MATERIALS AND METHODS

Virus: Strain K-47 of Chuzan virus [4] was used. The virus passaged 2 and 3 times in HmLu-1 cell cultures was employed for challenge inoculation for calves and detection of antibodies by virus neutralization (VN) tests, respectively. The virus passaged 12 times in BHK21 cell cultures after 3 serial passages in HmLu-1 cell cultures was used for the study of immunogenicity.

Vaccine and immunization: Roller culture bottles (110×285 mm) covered with a sheet of BHK21 cells received the virus (multiplicity of infection was approximately 0.001) and incubated at 37°C. The medium used after virus inoculation was Eagle's minimal essential medium containing 0.1% bovine albumin (fraction V), 10% tryptose phosphate broth and antibiotics. When an apparent cytopathic effect (CPE) appeared, the culture fluid was harvested and centrifuged at 3,000 rpm for 20 minutes to remove cell debris. The supernatant was treated with a final concentration of 0.05% formalin for 48 hrs or that of 0.1% β-propiolactone (BPL) for 24 hrs at 4°C. These inactivated viral suspensions were used as formalin-inactivated or BPL-inactivated vaccine.

For immunization, aluminum phosphate gel (APG) or an oil mixture (water-in-oil adjuvant supplied by Kyoto Biken Laboratories, Kyoto, Japan) was used as an adjuvant. A mixture of 9 volumes of the vaccine and 1 volume of APG or a mixture of 1 volume of the vaccine and 3 volumes of

the oil adjuvant was used for the immunization. A mixture of 9 volumes of the vaccine and 1 volume of phosphate buffered saline (PBS) was used as a control. These vaccines were inoculated intramuscularly into calves (11-month-old Japanese black) or guinea pigs (350 g) twice with a 3-week interval. Two calves and 5 guinea pigs were used for each vaccine. The dose of the immunogen was 3 m*l* for a calf and 0.5 m*l* for a guinea pig.

Challenge inoculation: Five weeks after the first inoculation of the vaccine (wpi), 4 immundized and 1 non-immunized calves were challenge-inoculated intravenously with 5 m*l* of a viral suspension ($10^{6.25}TCID_{50}$/m*l*).

Virus recovery from challenge-inoculated calves: Blood was collected daily after challenge inoculation and heparinized. The blood was centrifuged at 3,000 rpm for 20 minutes and the resultant plasma layer was collected. The sedimented red blood cells (RBC) were washed 3 times with PBS and the final pellet was resuspended in PBS to equal the original volume of the blood. The suspension was used for virus assay after lysis of the RBC by freezing and thawing. The plasma and RBC suspension were each inoculated into 4 tube cultures of BHK21 cells as described previously [4]. Detection of virus was determined by the appearance of a CPE after 2 serial subinoculations of each culture fluid into newly prepared cultures.

Virus titration: Undiluted or serial 10-fold dilution of viral suspensions, in amounts of 0.1 m*l*, were each inoculated into 4 tube cultures of BHK21 cells as described previously [4]. These cultures were incubated in a roller drum at 37°C. Infection of each culture was determined by the appearance of a CPE 5 to 7 days after inoculation and the $TCID_{50}$ was calculated.

VN test. This test was conducted by the serum dilution method with 100 $TCID_{50}$ of Chuzan virus in microplate cultures of BHK21 cells [4]. Two wells were used for each serum dilution. The antibody titer was expressed as the reciprocal of the highest serum dilution inhibiting CPE. Titers of 4 or higher were taken as positive.

RESULTS

Four tube cultures of each kind of cells were inoculated with Chuzan virus and incubated in a roller drum at 37°C. Each culture fluid was harvested, pooled and tested for infectivity when the maximum CPE was observed. As shown in Table 1, the virus grew well with an obvious CPE in cell lines originating in bovine, swine, monkey, and hamster tissues, and in the primary swine, guinea pig and hamster cells. Propagation was most pronounced in BHK21 cells and it was inapparent in the rabbit kidney cell line RK-13. Therefore. BHK21 cells were used for preparation of vaccine in subsequent experiments.

In roller culture bottles with a sheet of BHK21 cells, the virus growth reached the peak 3 to 4 days after virus inoculation, when the multiplicity of infection was 0.001 (Fig. 1). This condition for virus propagation was employed in subsequent experiments.

Curves of inactivation of Chuzan virus by formalin and BPL are shown in Figs. 2 and 3, respectively. In this experiment, the viral suspension treated with each reagent was harvested at designated times and tested for infectivity. The infectivity was inactivated by 48 hours after treatment with the final concentrations of 0.1 and 0.05% formalin at 4°C. Also, the infectivity decreased quickly and was undetectable 7 and 21 hours after treatment with 0.2 and 0.1% BPL at 4°C, respectively. A low level of infectivity, however, continued to be detected for 6 days after treatment with 0.05% BPL.

Samples of culture fluid of a roller bottle

Table 1. Propagation of Chuzan virus in various cell cultures

Cell designation	Cytopathic effect	Virus growth (log $TCID_{50}$/ml)
Bovine kidney (MDBK)	+	5.8
Bovine embryonic kidney (BEK-1)	+	3.8
Swine embryonic kidney (ESK)	+	5.5
Swine kidney (IB RS-2)	+	5.8
ditto (SK-L)	+	4.5
ditto (CPK)	+	6.0
Monkey kidney (VERO)	+	6.5
ditto (MA104)	+	5.8
Rabbit kidney (RK-13)	+	1.8
Hamster kidney (BHK21)	+	7.0
Hamster lung (HmLu)	+	6.0
Primary swine kidney cells	+	4.8
Primary swine testis cells	+	5.0
Primary guinea pig kidney cells	+	5.3
Primary hamster embryonic cells	+	5.5

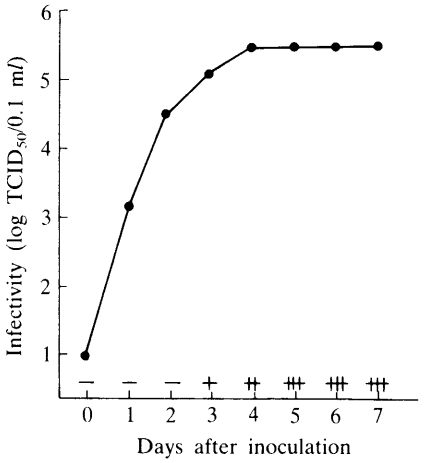

Fig. 1. Growth of Chuzan virus in a BHK21-cell culture at 37°C. −~卄 indicate degrees of cytopathic effect.

culture infected with the virus were harvested 3, 5, and 7 days after inoculation. The virus growth and appearance of CPE in the culture were similar to those in the culture shown in Fig. 1. The clarified fluid was inactivated with 0.05% formalin for 48 hours at 4°C. Undiluted fluid and serial 4-fold dilution of the fluid, in amounts of 0.5 ml, were each inoculated intramuscularly twice with a 3-week interval into 5 guinea pigs. Serum was collected 4 wpi and tested for VN antibody. These 3 culture fluids had similar antigenicity. That is, animals inoculated with undiluted culture fluid and 4-fold and 16-fold dilutions of the fluid showed antibody titers of 8–64, 4–16, 1–8, respectively.

The immunogenicity of formalin-inactivated vaccine and BPL-inactivated vaccine was compared. Each vaccine without an adjuvant was inoculated twice into guinea pigs as described above. Animals immunized with formalin-inactivated vaccine had a tendency to produce higher antibody titers than the animals immunized with BPL-inactivated vaccine (Table 2). Although the difference in antibody titer was not significant at 3 wpi, it was significant ($p=0.05$) at 4 wpi.

When guinea pigs were immunized with a formalin-inactivated vaccine which was mixed with different adjuvant, all animals developed detectable antibody by 3 wpi. The antibody titer reached the peak 4 or 5 wpi and then decreased gradually (Table 3). The average antibody titer of a group of animals was influenced by the adjuvant used. The group of animals immunized with

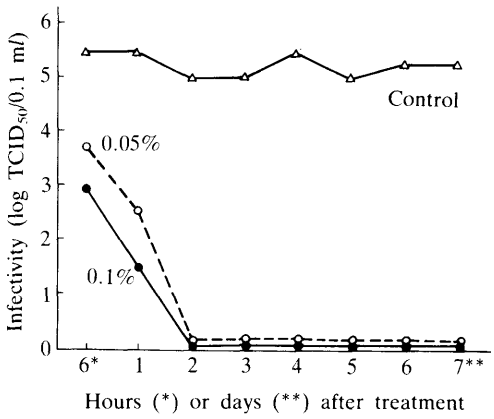

Fig. 2. Effect of formalin on infectivity of Chuzan virus at 4°C.

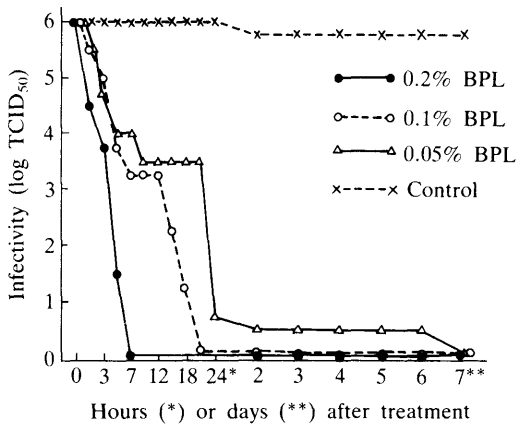

Fig. 3. Effect of β-propilolactone (BPL) on infectivity of Chuzan virus at 4°C.

Table 2. Comparison of immunogenicity of formalin-inactivated vaccine and BPL-inactivated vaccine

Inactivated with	Neutralizing antibody titer	
	Weeks after the first vaccination	
	3	4
Formalin	2–8[a] (4.57)	32–64 (56.23)
BPL	<1–8 (2.00)	8–64 (23.99)

a) Range of virus neutralizing antibody titers. Numbers in parentheses are the geometric means.

oil adjuvant (oil-adjuvant group; the word APG-adjuvant group is also used in this sense hereafter) always had significantly higher antibody titers than the control group. Also, the oil-adjuvant group showed a tendency to produce higher antibody levels than the APG-adjuvant group, although the difference was not so significant. There was no significant difference in antibody responses between the APG-adjuvant group and the control group by 6 wpi, but the titers at 8 and 10 wpi were significantly higher in the APG-adjuvant group than in the control group. Thus, production and maintenance of antibody were higher in the oil- and AGP-adjuvant groups than in the control group.

Formalin-inactivated vaccine mixed with APG or the oil adjuvant was each inoculated into 2 calves. Low levels of VN antibody to Chuzan virus were first detected 2 wpi and the titer rose to 4 to 8 at 3 wpi. A marked increase in antibody titers was observed after the second inoculation and the titers reached a maximum (64 and 128) at 4 or 5 wpi (Table 4). No clinical and hematological changes and no local reactions were observed after immunization.

After challenge inoculation, no calves showed a febrile response. However, the control calf manifested leukopenia 2 to 6 days after inoculation, whereas none of 4 immunized calves showed that sign (Table 5). Chuzan virus was detected in sera collected 2 and 11 days after inoculation and in RBC suspensions collected from 3 through 39 days, except the 28th and 35th days, from the control calf. In contrast, no viremia was detected in the immunized calves. Furthermore, VN antibody was first detected 2 weeks after the challenge inoculation in the control calf, and the titer reached a peak 3 weeks later, whereas no significant differences in antibody titers were observed between sera collected before and after challenge in immunized calves.

Table 3. Effect of adjuvant on immunogenicity of formalin-inactivated vaccine[a]

Group	Adjuvant	Neutralizing antibody titer: Weeks after the first vaccination 3	4	5	6	8	10
I	APG[b]	4[c]	32–128[c]	16–128	16–64	16–32	16–32
		4	74.13[d]	56.23	27.54	23.99	22.39
II	Oil	8–16	64–128	64–512	64–128	32–64	32–64
		10.47	112.20	169.82	91.20	45.71	45.71
III	Control (PBS)	2–8	32–64	32–64	8–32	4–16	4–16
		4.57	56.23	56.23	15.85	7.94	7.94
Significant difference between groups	I and II	**[e]	–	–	*	–	–
	I and III	–	–	–	–	**	*
	II and III	**	**	*	*	**	**

a) Each of 5 guinea pigs was inoculated intramuscularly twice with a 3-week interval with the vaccine mixed with the respective adjuvant.
b) Aluminum phosphate gel.
c) Range of virus neutralizing antibody titers.
d) Geometric mean.
e) –: No difference. *: p=0.05, **: p=0.001.

Table 4. Antibody responses of calves inoculated with formalin-inactivated vaccine mixed with APG or oil adjuvant[a]

Adjuvant used	calf No.	Weeks after the first vaccination 0	1	2	3	4	5
APG	979	<1[b]	<1	1	4	32	64
	980	<1	<1	1	8	64	64
Oil	981	<1	<1	2	4	32	128
	982	<1	<1	2	8	128	128

a) Inoculated with 3 m*l* of vaccine mixed with the respective adjuvant twice with a 3-week interval.
b) Virus-neutralizing antibody titer.

DISCUSSION

Vaccine prepared from Chuzan virus grown in BHK21 cell cultures had good immunogenicity in calves and guinea pigs. In guinea pigs, formalin-inactivated vaccine seemed to have better antigenicity than BPL-inactivated vaccine. Also, a vaccine mixed with the oil adjuvant had a tendency to stimulate the production of higher titers of antibody for a longer period than that mixed with APG adjuvant. If the net amount of the vaccine in the inoculum was taken into consideration (the net amount was 0.125 m*l* in 0.5 m*l* of inoculum mixed with the oil adjuvant and 0.45 m*l* in that mixed with APG adjuvant), the oil adjuvant will apparently be superior to the APG adjuvant from the viewpoint of immunogenicity.

The control calf showed leukopenia, viremia and a rise in antibody titers after challenge inoculation, whereas, none of 4 immunized calves showed them. In a separate study, all of 4 cattle used showed the signs of infection; i.e., leukopenia, persistent viremia and antibody responses after intravenous inoculation with the virus [5]. Therefore, although only one control calf was used in the present study, we believe

Table 5. Resistance of immunized calves to challenge with Chuzan virus

Group	Calf No.	Clinical finding		Viremia	Virus-neutralizing antibody titer at					
		Fever	Leukopenia		Challenge inoculation	Weeks after challenge 1	2	3	4	5
Immunized	979[a)]	–	–	–	64	64	64	32	32	32
	980	–	–	–	64	64	64	64	64	64
	981	–	–	–	128	128	64	64	NT[b)]	NT
	982	–	–	–	128	64	64	64	64	64
Control	983	–	+ (for 5 days)	+ (for 38 days)	<1	<1	16	256	256	256

a) Calves 979 and 980, and 981 and 982 were inoculated twice with a 3-week interval with vaccine mixed with APG and oil adjuvant, respectively. Challenge inoculation was given 2 weeks after the 2nd immunization with vaccine.
b) Not tested.

that immunization with the inactivated vaccine inhibited the propagation of Chuzan virus. Thus, it has become possible to develop an inactivated vaccine for Chuzan virus. However, further study is necessary to establish the stability and safety of the vaccine, duration of immunity and VN antibody titers that are required to prevent virus infection *in vivo*. Also, it remains to be determined whether inhibition of viremia prevents fetal infection, which is the most important manifestation of the disease. Prevention of viremia by the vaccine prevented transplacental infection of fetuses in the case of Akabane disease [3].

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REFERENCES

1. Goto, Y., Miura. Y., and Kono, Y. An epidemic of congenital abnormalities with hydrencephaly-cerebellar hypoplasia syndrome of calves in Japan: serological evidence for the etiologic role of Chuzan virus. a new orbivirus. *Am. J. Vet. Res.* (Submitted).
2. Goto, Y., Miura, Y., and Kono, Y. 1988. Epidemiological surveys of an epidemic of congenital abnormalities with hydranencephaly-cerebellar hypoplasia syndrome of calves occurring in 1985/86 and seroepidemiological investigation on Chuzan virus, a putative causal agent of the disease, in Japan. *Jap. J. Vet. Sci.* 50: 405–413.
3. Kurogi, H., Inaba, Y., Takahashi, E., Sato, L., Goto. Y., Satoda, K., Omori, T., and Hatakeyama, H. 1978. Development of inactivated vaccine for Akabane disease. Natl. Inst. *Anim. Health Q.* 18: 97–108.
4. Miura, Y., Goto, Y., Kono, Y. Isolation of Chuzan virus, a new member of the palyam subgroup of genus Orbivirus, from cattle and *Culicoides oxystoma* in Japan. *Am. J. Vet. Res.* (submitted).
5. Miura, Y., Goto, Y., Kubo, M., and Kono. Y. 1988. Pathogenicity of chuzan virus, a new member of Palyam subgroup of genus *Orbivirus* for cattle. *Jpn. J. Vet. Sci.* 50: 632–637.

要　約

Chuzan ウイルス不活化ワクチン開発のための基礎試験：後藤義之・三浦康男・甲野雄次（家畜衛生試験場九州支場）——Chuzan ウイルスは BHK21 細胞培養中で最もよく増殖し，その培養液をホルマリンで不活化した抗原はモルモット及び牛で高い抗原性を示した．油性アジュバントまたは燐酸アルミニウムゲルの添加により抗体産生は促進され，免疫牛は攻撃接種後も白血球減少や持続性ウイルス血症を示すことなく耐過した．