

# 羊を用いた牛白血病ウイルスの能動免疫および受動免疫による感染防御試験

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## Protection against Bovine Leukemia Virus Infection in Sheep by Active and Passive Immunization

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**ABSTRACT.** The protective effect of active and passive immunization against bovine leukemia virus (BLV) infection was tested in sheep. Eight sheep were immunized 3 times at 2-week intervals with concentrated BLV antigen treated with Triton X-100. Two weeks after the last immunization, when antibody titers to glycoprotein (gp) (gp antibody) were 32 to 256, 4 of the 8 immunized sheep were challenged with infected sheep blood and remained uninfected. These 8 sheep were challenged or rechallenged when their gp antibody titers fell to 1 to 8, and all animals were infected. Of 10 sheep immunized passively with various doses of immunoglobulin obtained from infected sheep serum, animals that had gp antibody titers of 64 showed protection against challenge inoculation. To sum up the relationship between serum antibody titers and protective effect, all sheep that had gp antibody titers of 64 or more and half the sheep that had titers of 32 showed resistance to infection. Resistance was observed in only one of the 13 sheep that had titers of 16 or less.—**KEY WORDS:** Bovine leukemia virus, Immunization, Resistance, Vaccination.

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Slaughter of bovine leukemia virus (BLV) antibody-positive cattle or their segregation from the negative cattle has been shown to be the most effective program for eradication of BLV [6, 10, 19]. However, such a program seems to be impractical in districts that are heavily contaminated with BLV for economic reasons. Therefore development of an effective vaccine to prevent BLV infection is necessary.

Vaccination against BLV has given partial or complete protection in cattle [11, 12, 15, 16] and sheep [13, 15]. However, the duration of protective effect and the antibody levels required for protection have not yet been investigated.

The purpose of this investigation was to determine the conditions for protection against BLV infection in sheep. The effect of passive immunization with specific immuno-

globulin (IgG) was also investigated to determine the mechanisms of the protection.

### MATERIALS AND METHODS

*Antigen for immunization (immuogen):* Fetal lamb kidney cells persistently infected with BLV [18] were grown in Eagle's minimum essential medium containing 10% tryptose phosphate broth and 3% sheep serum. The culture fluid was centrifuged at 4,000×g for 30 minutes at 4°C. The supernatant was mixed with ammonium sulphate (0.318 g/ml), kept in an ice bath for 1 hour and centrifuged at 4,000×g for 30 minutes at 4°C. The precipitate was dissolved in a minimal amount of phosphate buffered saline (PBS) solution at pH 7.2, dialysed in the PBS solution, and concentrated to 1/100 of the volume of the original culture fluid. The concentrate was mixed with 0.1% Triton X-100 (final). This material, which had BLV-glycoprotein (gp) antigenicity of 64 to 128

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units and BLV-protein (p) antigenicity of 256 to 512 units in the agar gel immunodiffusion (ID) test [9], was used as immunogen.

*Immunization and challenge inoculation:* Eight sheep (5 months old) were injected intramuscularly 3 times, at 2-week intervals, with 2 ml of an immunogen-Freund's complete adjuvant mixture. Two weeks after the last injection, 4 of the immunized and 4 nonimmunized sheep were challenge-inoculated intradermally with 50  $\mu$ l of heparinized BLV-infected sheep blood, which contained approximately  $2 \times 10^5$  lymphocytes and developed 10–50 syncytia in repeated *in vitro* tests. The remaining 4 immunized animals were observed as controls. All immunized sheep were challenged again as described above 45 weeks after the first immunization (WPI).

*Passive immunization with IgG:* Sera collected from 1 BLV-infected sheep and 2 BLV-immunogen injected sheep after BLV infection were pooled. The IgG fraction was collected from the pooled sera by 3 repeated cycles of precipitation procedures using one-third saturation with ammonium sulphate. The final precipitate was suspended in PBS solution equal in volume to the original serum. Antibody titers of the suspension to gp and p antigens were 512 and 64, respectively.

Various volumes of IgG solution were injected intraperitoneally into sheep as shown in Results. The animals were challenged with BLV-infected sheep blood 2 or 3 days after the IgG was given as described above.

*Determination of serum antibody titer:* This was done by the ID test with gp and p antigens as described previously [9]. Sera were collected from test animals at 1-day to 2-week intervals. The average antibody titer of a group is shown as the geometric mean.

*Test for BLV in lymphocytes:* Infection of test animals with BLV was confirmed by *in vivo* and *in vitro* assays for BLV in lymphocytes isolated from peripheral blood of the animals. For *in vivo* assay, 1-year-old sheep

were inoculated subcutaneously with  $5\text{--}20 \times 10^7$  lymphocytes and examined by ID tests for 6 months. One animal was used for each test. For *in vitro* assay, a syncytium induction assay (SIA) with bovine embryonic spleen cells was used. Details of the method were given previously [8].

## RESULTS

*Lack of infectivity of the immunogen:* Three sheep inoculated subcutaneously with 1 ml of immunogen showed neither detectable immunological response nor infection in the SIA for 1 year.

*Protection against infection after inoculation with immunogen:* In 8 sheep inoculated with the immunogen, antibodies against gp and p antigens (gp and p antibodies) appeared 2 WPI and reached the peak titers 3 to 6 WPI (Figs. 1 and 2). The highest antibody titers were 32 to 256 (Ave. 108) to gp antigen and 64 to 1,024 (Ave. 181) to p antigen. These titers decreased gradually thereafter. Antibody titers to p antigen were always higher than gp antibody titers in all sheep during the observation period.

Four of the 8 immunized sheep and 4 nonimmunized control sheep were inoculated with the blood of BLV-infected sheep (challenge inoculation) 5 WPI. Challenge-inoculated immunized sheep had gp antibody titers of 32 to 128 at that time (Table 1). The nonimmunized control sheep changed to seropositive between 4 and 7 weeks after challenge inoculation (WPC) and BLV was detected in all sheep by SIA. On the other hand, the change in antibody titers in challenge-inoculated immunized sheep was quite similar to that in control immunized sheep (Figs. 1 and 2, Table 1). The gp antibody titers decreased gradually after challenge inoculation and were 1 to 4 at 44 WPI. BLV could not be isolated from any immunized sheep by *in vivo* tests 0, 17 and 33 WPC (Table 1).

Table 1. Immune response and virus isolation in immunized sheep after challenge inoculation with infected sheep lymphocytes at 5 weeks after the first immunization

Exp. group	Sheep No.	gp antibody titer at		Virus isolation at		
		challenge	7 WPC	0 WPC	17 WPC	33 WPC
Immunized control	60	128	8	- <sup>a)</sup>	-	-
	61	256	64	-	-	-
	62	128	32	-	-	-
	63	128	32	-	-	-
Immunized and challenge inoculated	65	64	16	-	-	-
	66	128	32	-	-	-
	67	64	16	-	-	-
	69	32	4	-	-	-
Challenge-inoculated control	70	-	2	NT <sup>b)</sup>	+ <sup>c)</sup>	
	71	-	1	NT	+	
	72	-	32	NT	+	
	73	-	4	NT	+	

a) *in vivo*.

b) Not tested.

c) *in vitro*.

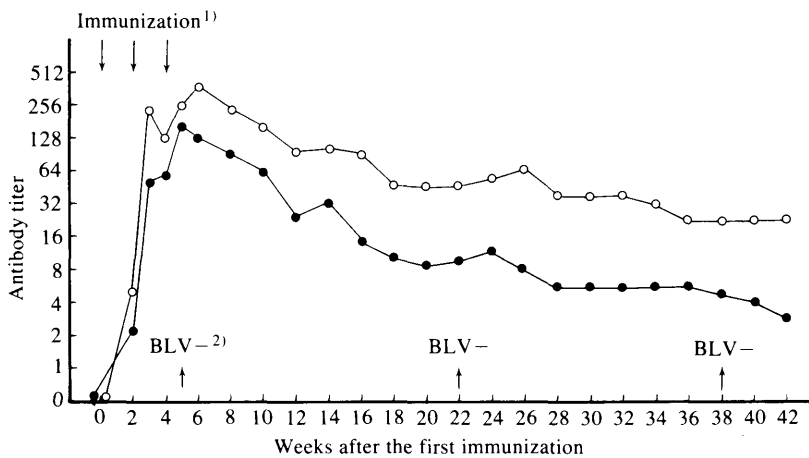


Fig. 1. Immune responses and virus isolation in sheep after repeated immunization. The average antibody titers of 4 sheep are shown. 1) A mixture of 1 ml each of BLV antigen and Freund's complete adjuvant was administered intramuscularly. 2) Isolation of BLV was negative by *in vivo* test. —○—, —●—: p and gp antibody titers, respectively.

Four challenge-inoculated immunized sheep and 4 control immunized sheep were challenge-inoculated again with infected sheep blood 45 WPI when gp antibody titers of the sheep were 1 to 8. Marked increase in gp antibody titers was observed 2 WPC and

the increase continued until 6 WPC (Fig. 3). During this stage, gp antibody titers increased 64-fold on average over the prechallenge inoculation level, but the increase in p antibody titers was only about twofold. BLV was isolated by *in vivo* tests from all sheep 10

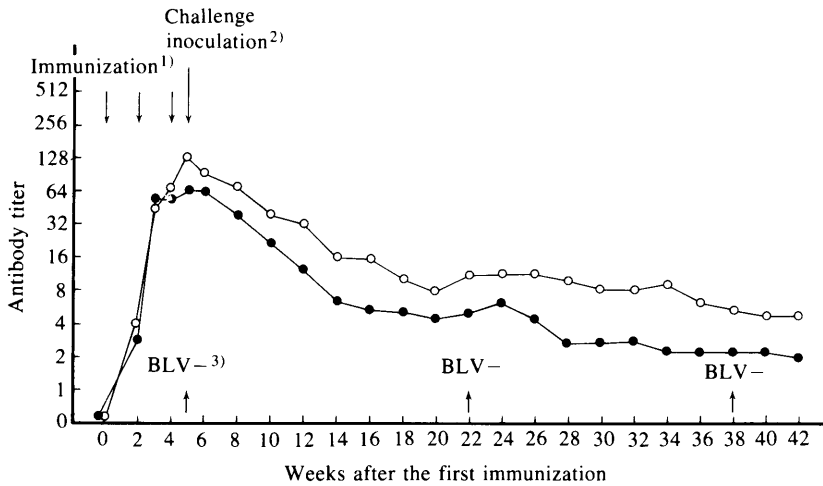


Fig. 2. Immune responses and virus isolation in sheep after repeated immunization and challenge inoculation with infected sheep blood. The average antibody titers of 4 sheep are shown. 1) See footnote 1) of Fig. 1. 2) 50  $\mu$ l of infected sheep blood was inoculated intradermally. 3) See footnote 2) of Fig. 1. —○—, —●—: p and gp antibody titers, respectively.

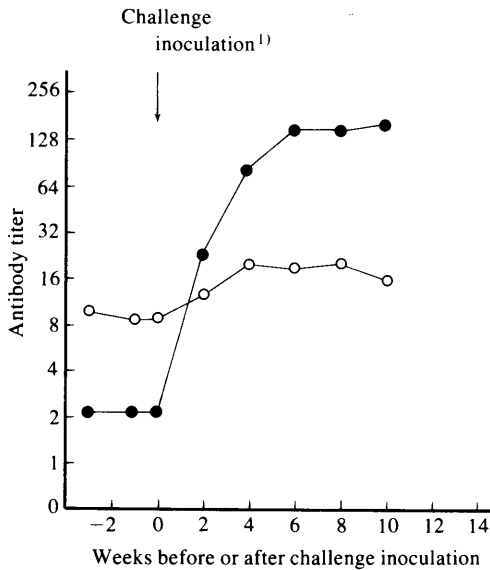


Fig. 3. Immune responses in immunized sheep after challenge inoculation at 45 weeks after the first immunization. The average antibody titers of 8 sheep are shown. 1) See footnote 2) of Fig. 2. —○—, —●—: p and gp antibody titers, respectively.

WPC. On the other hand, seroconversion in nonimmunized challenge control sheep was as late as 6 to 8 WPC, and the gp antibody titer remained at a barely detectable level for

6 weeks after the seroconversion (Table 2).

*Protection against infection by IgG injection:* Ten sheep were injected with 30 to 300 ml of IgG solution intraperitoneally. Passive gp antibody titers reached the highest levels, i.e., 1 to 64 depending on the dose of IgG solution, 2 to 3 days after the injection, when the challenge inoculation was performed. These sheep were observed for 18 WPC (Table 3).

Typical changes in antibody titers in IgG-injected and control sheep are shown in Fig. 4. In 2 sheep that had antibody titer of 64, the antibodies decreased gradually, became barely detectable at 10 WPC and disappeared thereafter (Fig. 4a). In 1 sheep that had an antibody titer of 32, the titer decreased to an undetectable level between 10 and 12 WPC and then increased (Fig. 4b). One animal that had a passive antibody titer of 16 died 13 WPC, so the final results could not be obtained, although the antibody was undetectable at the time. In 2 sheep that had antibody titers of 4, the titer fell to a barely detectable level and then remained at the minimal detectable level during the observation period (Fig. 4c).

Table 2. Immune response and virus isolation in immunized sheep after challenge inoculation with infected sheep lymphocytes at 45 weeks after the first immunization

Exp. group	Sheep No.	gp antibody titer at			Virus isolation at
		challenge	2 WPC	8 WPC	10 WPC
Immunized and challenge inoculated	60	1	32	64	+ <sup>a)</sup>
	61	2	32	256	+
	62	8	32	256	+
	63	4	8	32	+
	65	4	64	128	+
	66	4	64	256	+
	67	1	16	256	+
	69	1	64	256	+
Challenge-inoculated control	88	—	—	1	+ <sup>b)</sup>
	89	—	—	1	+
	90	—	—	1	+
	91	—	—	1	+

a) *in vivo*.

b) *in vitro*.

Table 3. Immune response and virus isolation after challenge inoculation in sheep injected with igG

Exp. group	Sheep No.	gp antibody titer at			Virus isolation at
		challenge	10 WPC	18 WPC	18 WPC
IgG injected	76	64	1	—	— <sup>a)</sup>
	80	64	1	—	—
	81	32	—	1	+
	79	16	1	NT <sup>b)</sup>	NT
	77	4	1	1	+
	78	4	1	1	+
	58	1	8	NT	+
	59	1	8	NT	+
	74	1	16	NT	+
	75	1	—	—	—
	Control	70	—	8	16
71		—	4	8	+
72		—	32	64	+
73		—	1	4	+
82		—	—	1	+
84		—	—	4	+

a) *in vivo*.

b) Not tested.

c) *in vitro*.

Of 4 sheep that had antibody titers of 1, 3 showed an increase in the titer 2 to 6 WPC, the remaining 1 sheep became seronegative 3 WPC and remained so thereafter.

BLV was isolated by *in vivo* cultures from

6 seropositive cases but not from 3 seronegative cases at 18 WPC.

*Relationship between serum gp antibody titer and protective effect:* The relationship between serum antibody titer and protective

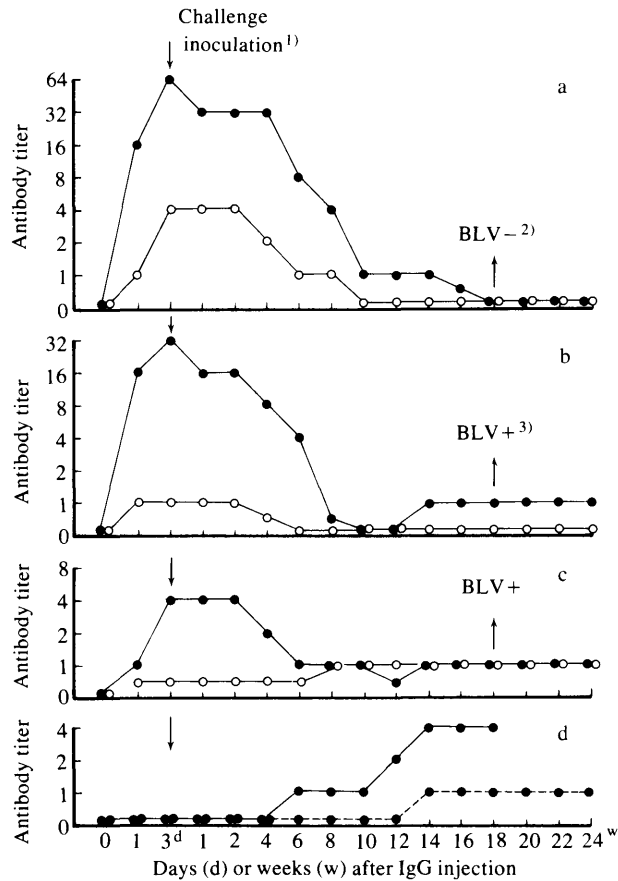


Fig. 4. Changes in antibody titers in sheep after IgG injection and challenge inoculation. p and gp antibody responses in sheep Nos. 76, 81 and 77 are shown in Graphs a, b and c, respectively. gp antibody responses in 2 control sheep (Nos. 73 and 82) are shown in the bottom graph, d. 1) See footnote 2) of Fig. 2. 2) and 3) Indicate BLV-isolation negative and positive, respectively, by *in vivo* tests. —○—, —●— : p and gp antibody titers, respectively.

Table 4. Resistance of animals with various serum gp antibody titers against challenge inoculation with infected sheep blood

	gp antibody titer								
	—	1	2	4	8	16	32	64	128
No. tested	10	7	1	5	1	1 <sup>a)</sup>	2	3	1
No. infected	10	6	1	5	1		1	0	0
Protection rate (%)	0	14.3	0	0	0		50	100	100

a) No final result was obtained since the animal tested died before the end of the observation period.

effect in all sheep used in the experiment is summarized in Table 4. All sheep that had gp antibody titers of 64 or more and half of the sheep that had titers of 32 showed resistance to infection. Resistance was observed in only one of the sheep that had gp antibody titers of 16 or less.

#### DISCUSSION

Protection against BLV infection in sheep was established after repeated infection of crude BLV antigen, as revealed by bioassay using sheep. The protection seemed to be mediated by specific IgG since the protective effect was transferred to nonimmunized sheep by IgG obtained from immunized sheep serum. It has been reported that the expression of the BLV genome in infected lymphocytes is blocked by a non-IgG protein in plasma but not in serum of BLV-infected cattle [4, 5]. The IgG used in our experiments was prepared from serum, so the participation of non-IgG protein for protection can be denied.

Since crude viral antigen was used as an immunogen, both gp and p antibodies were produced in immunized sheep. The protective effect, however, might have been conferred by gp antibody as already shown by *in vitro* and *in vivo* experiments [3, 13, 14].

ID antibody titers of 64 were required to prevent BLV infection mediated by infected lymphocytes. The titer was markedly higher than the complement-fixing titer of 8 that was considered by Onuma *et al.* [13] to be necessary for protection against BLV infection. This discrepancy might be caused by a difference in the methods for measuring antibody titers or a difference in the materials used for challenge inoculation (Onuma *et al.* used lymphocytes of infected cattle).

BLV infect cattle during the summer and autumn when blood-sucking insects are active [2]. Therefore, maintenance of a high antibody level for at least 4 months is necessary for prevention of BLV infection in middle

latitudes. However, in our study, the duration of protective antibody titers of 64 or more ranged from 22 to 71 days, with an average of 50 days, except in an animal whose highest antibody titer was 32. Therefore, further experiments on maintaining high antibody titers for long periods are required.

As one of the defects of vaccination against BLV infection that is characterized by life-long persistent cell associated viremia, difficulty in distinguishing antibodies induced by infection from those induced by vaccination is pointed out. In animals naturally infected with BLV, gp antibody titers are consistently higher than p antibody titers [7, 9]. In contrast, p antibody titers were higher than gp antibody titers in animals immunized with crude BLV antigen. Therefore, discrimination of antibodies raised by vaccination seems to be possible by comparing the two antibodies.

Sudden and marked increase in gp antibody titers was observed in immunized sheep that had shown a fall in antibody titers, after challenge inoculation with 50  $\mu$ l of blood obtained from infected sheep. The blood contained as few lymphocytes as approximately  $2 \times 10^5$  cells. Since such an increase in antibody titers was not observed in nonimmunized control sheep, the increase was thought to be the result of a booster effect. It seems that the booster effect was caused by direct interaction between infected lymphocytes and specific memory B cells, since release of mature virus from infected lymphocytes is inhibited by antibody [1, 17]. This fact suggests the possibility that infection of susceptible animals with BLV might be established by direct contact between lymphocytes or lymphoblasts of the host animal and infected lymphocytes which were transferred by blood-sucking insects.

#### REFERENCES

1. Baliga, V., and Ferrer, J. F. 1977. Expression of the bovine leukemia virus and its internal antigen



- in blood lymphocytes. *Proc. soc. Exp. Biol. Med.* 156: 388-391.
2. Bech-Nielsen, S., Piper, C. E., and Ferrer, J. F. 1978. Natural mode of transmission of the bovine leukemia virus: Role of blood-sucking insects. *Am. J. Vet. Res.* 39: 1089-1092.
  3. Driscoll, D. M., Onuma, M., and Olson, C. 1977. Inhibition of bovine leukemia virus release by antiviral antibodies. *Arch. Virol.* 55: 139-144.
  4. Gupta, P., and Ferrer, J. F. 1982. Expression of bovine leukemia virus genome is blocked by non-immunoglobulin protein in plasma from infected cattle. *Science* 215: 405-406.
  5. Gupta, P., Kashmiri, S. V. S., and Ferrer, J. F. 1984. Transcriptional control of the bovine leukemia virus genome: role and characterization of a non-immunoglobulin plasma protein from bovine leukemia virus-infected cattle. *J. Virol.* 50: 267-270.
  6. Jazbec, I., Gregorovic, V., and Skusek, F. 1982. Extent, diagnosis and eradication of enzootic bovine leukosis in Socialist Republic of Slovenia (Yugoslavia). *Curr. Top. Vet. Med. Anim. Sci.* 15: 384-395. Fourth Int. Symp. Bovine Leukosis (Straub, O. C. ed.).
  7. Kono, Y., Hatakeyama, H., Ishikawa, H., and Sentsui, H. 1981. Antibody titers in cattle clinically and subclinically infected with bovine leukemia virus. *Vet. Microbiol.* 6: 167-170.
  8. Kono, Y., Sentsui, H., Arai, K., Fujigaki, A., Enomoto, C., Iwasaki, H., and Ishida, H. 1983. Serological methods to detect calves infected in utero with bovine leukemia virus. *Jpn. J. Vet. Sci.* 45: 453-461.
  9. Kono, Y., Sentsui, H., Miyamoto, T., Morozumi, K., and Sakamoto, Y. 1982. Changes in antibody titers in cattle infected clinically and subclinically with bovine leukemia virus. *Int. J. Cancer* 30: 655-657.
  10. Mammerickx, M., Corman, A., Burny, A., Dekegel, D., and Portelle, D. 1978. Eradication of enzootic bovine leukosis based on the detection of the disease by the GP immunodiffusion test. *Ann. Rech. Vet.* 9: 885-894.
  11. Miller, J. M., and Van Der Maaten, M. J. 1978. Evaluation of an inactivated bovine leukemia virus preparation as an immunogen in cattle. *Ann. Rech. Vet.* 9: 871-877.
  12. Miller, J. M., Van Der Maaten, M. J., and Schmerr, M. J. F. 1983. Vaccination of cattle with binary ethylenimine-treated bovine leukemia virus. *Am. J. Vet. Res.* 44: 64-67.
  13. Onuma, M., Hodatsu, T., Yamamoto, S., Higashihara, M., Masu, S., Mikami, T., and Izawa, H. 1984. Protection by vaccination against bovine leukemia virus infection in sheep. *Am. J. Vet. Res.* 45: 1212-1215.
  14. Onuma, M., Watarai, S., Mikami, T., and Izawa, H. 1980. Cell fusion activity of bovine leukemia virus. *J. Gen. Virol.* 48: 421-424.
  15. Parfanovich, M. I., Zhdanov, V. M., Lazarenko, A. A., Momm, E. M., Simovart, Yu. A., Parakin, V. K., and Lemesch, V. M. 1983. The possibility of specific protection against bovine leukemia virus infection and bovine leukemia with inactivated BLV. *Br. Vet. J.* 139: 137-146.
  16. Patrascu, I. V., Coman, s., Sandu, I., Stiube, P., Munteanu, I., Coman, T., Ionescu, M., and Popescu, D. 1980. Specific protection against bovine leukemia virus infection conferred on cattle by Romanian inactivated vaccine BL-Vacc-RO. *Rev. Romanie Med. Virol.* 31: 95-102.
  17. Stock, N. D., and Ferrer, J. F. 1972. Replicating C-type virus in phytohemagglutinin-treated buffy coat cultures of bovine origin. *J. Natl. Cancer Inst.* 48: 985-996.
  18. Van Der Maaten, M. J., and Miller, J. M. 1975. Replication of bovine leukemia virus in monolayer cell cultures. *Bibl. Haematol.* 43: 360-362.
  19. Yoshikawa, T., Yoshikawa, H., Koyama, H., and Tsubaki, S. 1982. Preliminary attempts to eradicate infection with bovine leukemia virus from a stock farm in Japan. *Jpn. J. Vet. Sci.* 44: 831-834.

## 要 約

羊を用いた牛白血病ウイルスの能動免疫および受動免疫による感染防御試験：甲野雄次・新井啓五・泉対 博・松川俊一・糸原重美（農林水産省家畜衛生試験場）——羊を用いて牛白血病ウイルス（BLV）の感染防御実験を実施した。Triton X-100 により不活化した 100 倍濃縮 BLV 培養液をフロイド完全アジュバントとともに 8 頭の羊に 2 週間隔で 3 回筋肉内接種した。最終免疫から 2 週後、すなわち gp 抗体価が 1 : 32 ~ 1 : 256 に達した時に、4 頭の免疫羊に 50  $\mu$ l の BLV 感染羊血液を皮内接種したが感染は成立しなかった。この 4 頭を含めた 8 頭の免疫羊の抗体価が 1 : 1 ~ 1 : 8 に低下した時、同一方法で再び攻撃接種を行った。その結果、すべての羊は抗体価の著明な上昇を示し、同時に BLV も分離された。BLV 感染羊の血清から分離した種々の量の免疫グロブリンを、健康羊に腹腔内接種した後攻撃接種を行った結果、1 : 64 の抗体価を持った個体では感染防御が成立した。実験に用いたすべての羊につき感染防御の成立と液性抗体価の関係を調べた結果、1 : 64 以上の抗体価を持つすべての羊および 1 : 32 の抗体価を持つ羊の半数で感染防御が成立したが、1 : 16 以下の抗体価を持つ個体では 1 頭が感染防御を示したにすぎなかった。