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The Screening of Cattle with Potential for Developing Leukemia by Using Monoclonal Antibody against Bovine Leukemia Cells

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Tumor cells from cattle with enzootic bovine lymphosarcoma (EBL) have a tumor-associated antigen (TAA) which is distinct from bovine leukemia virus (BLV)-induced antigens. We were able to sacrifice 8 TAA-positive cattle with no clinical signs of EBL and to examine whether or not they had gross or histological tumors. At necropsy, 4 animals had tumors macroscopically. Three animals had no tumors histologically but had initial lesions showing follicular hyperplasia and had the TAA on affected lymph nodes. The remaining one showed medullary hyperplasia in the spleen but there were no findings of tumors. These results suggest that most BLV-infected cattle which are TAA-positive but have no clinical signs of EBL, do have tumors and have a higher potential for developing EBL in the future when compared to BLV-infected but TAA-negative cattle.

Key words: Tumor-associated antigen — Bovine leukosis — Monoclonal antibody — Bovine leukemia virus

Enzootic bovine lymphosarcoma (EBL) is a B cell lymphoma caused by bovine leukemia virus (BLV). Specific tumor-associated antigen (TAA)[1] was detected on peripheral blood lymphocytes (PBL) from EBL cows as well as on tumor cells by using a monoclonal antibody against TAA.[2] Since the monoclonal antibody, c143, reacted positively only with tumor cells but not with BLV-negative normal adult bovine lymphoid cells, the antibody has been used as a diagnostic tool for the detection of EBL cows.[3] The TAA identified by c143 was also detected on PBL from most of the BLV-infected cows with lymphocytosis, and from some of the BLV-infected cows with no clinical signs of EBL.[3] We are making long-term observations of TAA-positive healthy carriers to see if any of them develop EBL. During the course of the observations, two TAA-positive cows with no clinical signs of EBL at the time of testing became leukemic approximately one and 3 years later, respectively. Based on these findings we speculated that cows with the potential to develop EBL in the future might be screened by using c143. In the present studies, we sacrificed 8 TAA-positive but clinically normal animals and examined them to establish whether or not they have gross or histological tumors.

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MATERIALS AND METHODS

Animals During the follow-up of the TAA-positive but clinically normal cattle, we were able to sacrifice the following 8 cattle for histopathological observations. Five of them (Nos. 1 to 5) were Holstein bulls aged 7 to 11 years old and one was a 6-year-old Japanese Black bull (No. 8). The remaining two (Nos. 6 and 7) were Holstein cows, aged about 8 years old. These 8 BLV-infected animals were positive for TAA on their PBL but were clinically normal at the time of sacrifice.

Detection of TAA The TAA was detected on PBL and lymph nodes by either the complement-dependent antibody cytotoxicity (CDAC) test or the immunofluorescence (IF) test using monoclonal antibody c143[2] and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG. The methods for the CDAC and IF tests were described previously.[1, 2, 4] The cytotoxic index (CI) was calculated by means of the following formula: CI=

(% viable cells in control wells − % viable cells in test sample)/(% viable cells in control wells) × 100. A CI value greater than 31.7 was considered positive.[2]

Double Staining To determine whether atypical lymphocytes have TAA or not, double staining was performed. Acetone-fixed cells were incubated with c143, washed and then stained with FITC-conjugated anti-mouse IgG. After IF staining, the cells were stained with Giemsa.

Detection of BLV At the time of necropsy, fresh materials were obtained in a culture medium, then made into a single suspension of cells and cultured with 5 μg/ml of concanavalin A for 2 to 3 days.[5] After cultivation, BLV antigen was detected by means of the IF test using anti-BLV serum.[6]

Detection of Bovine Leukemia Provirus A hybridization probe was obtained from molecularly cloned λBLV-1[7] through *Pvu* II and *Sac* I digestion, labeled with ^{32}P by nick translation and used as the hybridization probe as described previously.[8] High-molecular-weight bovine DNA was extracted from various lymph nodes and PBL.[9] The extracted DNA was digested with *Eco* RI and provirus was detected by the method of Southern.[10]

RESULTS

Hematological results on 8 cattle tested at the time of sacrifice are shown in Table I. Cows No. 5, No. 6 and No. 7 showed lymphocytosis and had relatively higher percentages of atypical lymphocytes at the time of sacrifice. The evolution of the TAA in PBL from 4 cattle (Nos. 1 to 4) was examined by means of the CDAC test and the results were expressed as CI values. On May 24th, 1984, the CI values of these 4 animals were negative, but one (No. 4) converted to positive about 15 months later (Table II). By December, 1985, all of the other 3 animals had converted to positive. These 4 animals were sacrificed at the times indicated in Table II, to determine whether they had tumors or not. The results on the expression of TAA in PBL

Table I. Hematological Findings in Cattle Tested

	Lymphocyte count (/μl)	Lymphocytes (%)	Atypical lymphocytes (%)
No. 1	2,250	55	1
No. 2	1,180	38	4
No. 3	2,730	47	4
No. 4	1,037	15	1
No. 5	8,800	56	9.5
No. 6	14,393	50.5	9
No. 7	9,585	67.5	7.5
No. 8	7,000	78.6	NT

Table III. Expression of TAA on Peripheral Blood Lymphocytes and Pathological Observations at Necropsy

Test animal	Expression of TAA (CI)	Presence of tumor	
		Gross	Histology
No. 1	40.7	No	Initial lesion
No. 2	48.8	No	Initial lesion
No. 3	47.1	Yes	Yes
No. 4	39.3	Yes	Yes
No. 5	38.8	No	Initial lesion
No. 6	52.0	No	No[a]
No. 7	65.1	Yes	Yes
No. 8	47.6	Yes	Yes

CI: Cytotoxic index.
a) Medullary hyperplasia in the spleen was noted.

Table II. Evolution of TAA-positive Cells on Peripheral Blood Lymphocytes from BLV-infected Cattle

	Date tested					
	May 24/84	Aug. 7/85	Nov. 26/85	Dec. 20/85	Dec. 24/85	Jan. 3/86
No. 1	8.5[a]	23.4	ND	37.8	ND	40.7[b]
No. 2	0	ND	ND	40.7	48.8[b]	
No. 3	11.7	14.9	40.5	52.1	47.1[b]	
No. 4	0	37.5	39.3[b]			

a) Cytotoxic index determined by CDAC test.
b) All cattle were killed for histopathological examinations at this time.
ND: Not determined.

at the time of sacrifice and existence of tumors at necropsy are shown in Table III. At the necropsy of No. 4, tumor masses were observed in the abomasum (Fig. 1 a, b), surrounding the kidney and heart. In the case of No. 3, tumor masses were also found in the abomasum. Mesenteric and iliac lymph nodes were enlarged and the TAA was detected by the IF test (Fig. 2). In cases No. 1, No. 2 and No. 5, the CI values in their PBL were 40.7, 48.8 and 38.8, respectively, and no gross tumor was found in any lymph node or tissue. No histological tumor was found in any lymph node, but initial lesions showing follicular hyperplasia were observed in some lymph nodes (Fig. 3a). These lymph nodes

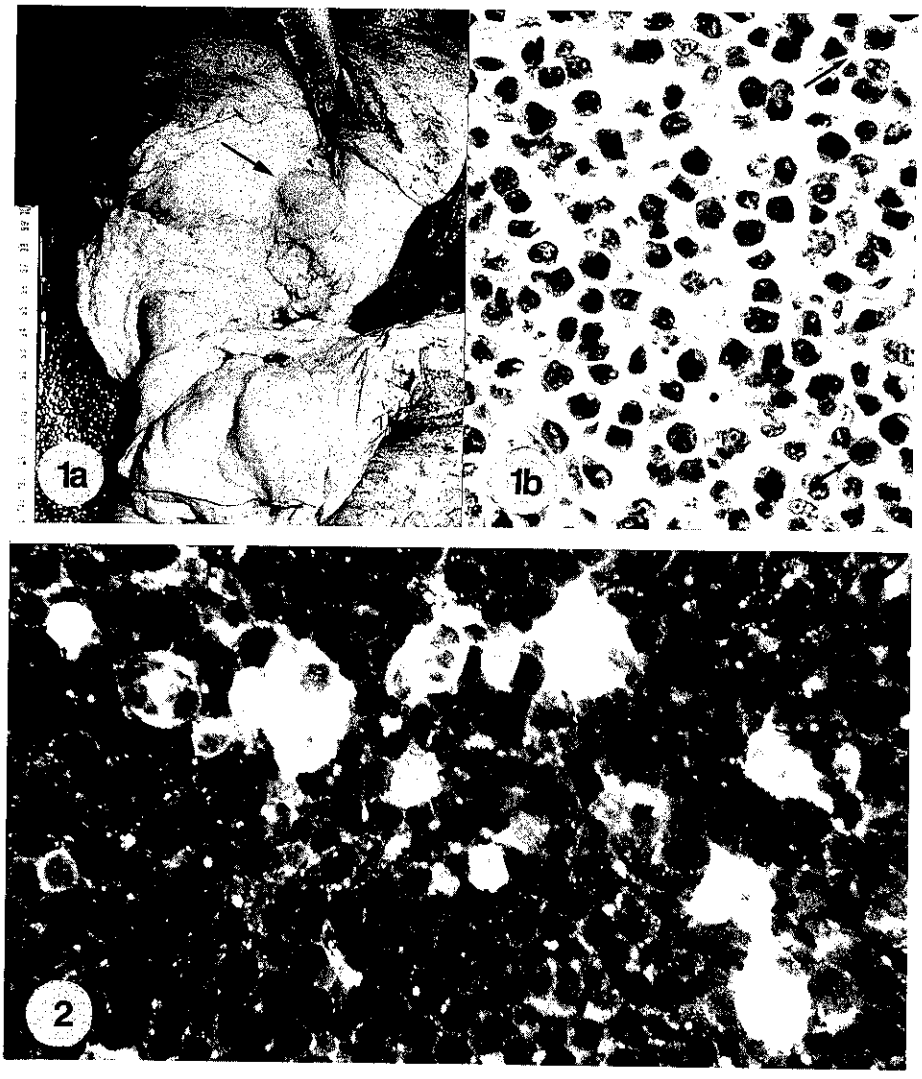

Fig. 1. Macroscopic and light microscopic observations of the tumor in the abomasum of case No. 4 at necropsy. a: A nodular tumorous region (↑) within the pylorus well. b: Hematoxylin-eosin-stained section (×440). Diffuse proliferation of neoplastic cells and mitotic figures (↑) are frequently observed.

Fig. 2. Stamp smear of mesenteric lymph node of case No. 4 stained positively with cl43 and FITC-conjugated anti-mouse IgG (×800).

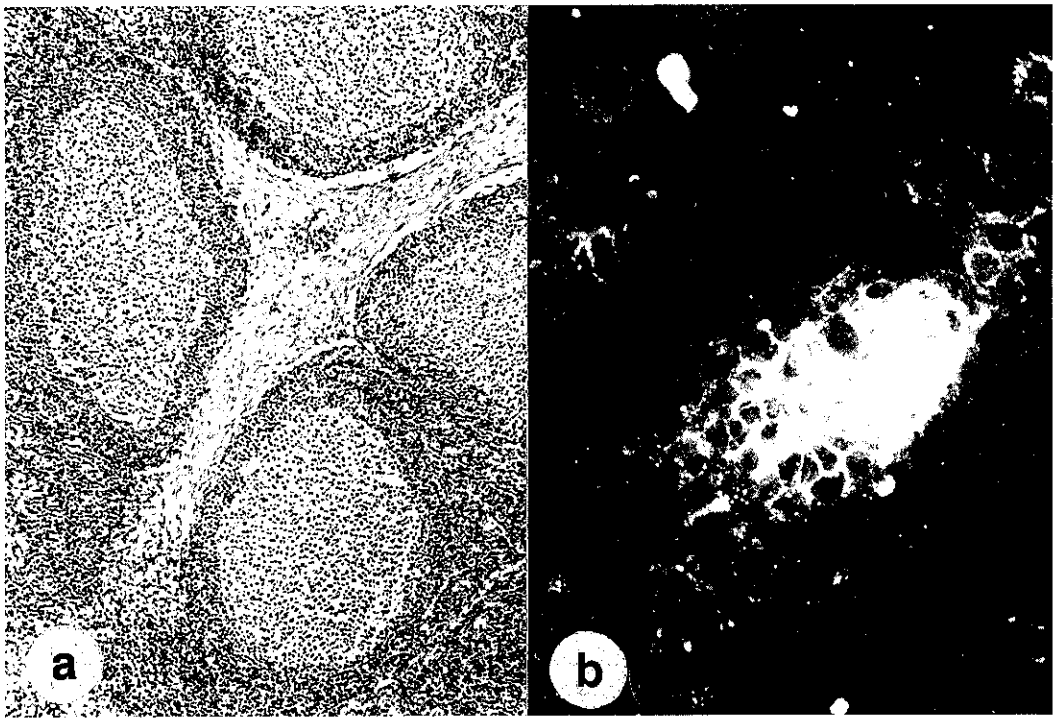

Fig. 3. Light microscopic observation and immunofluorescence staining of lymph node from case No. 5. a: Hematoxylin-eosin-stained section (×70). Initial lesions showing follicular hyperplasia. b: Indirect immunofluorescence test using c143 and FITC-conjugated anti-mouse IgG (×800).

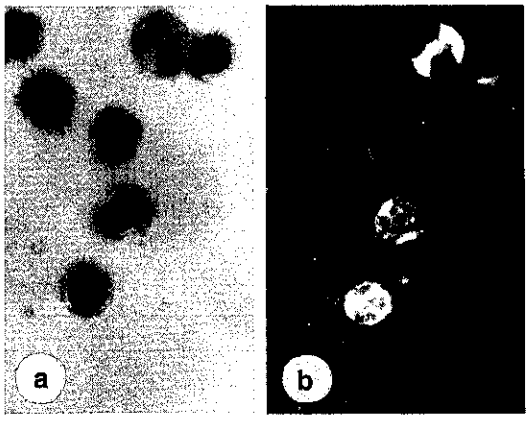

Fig. 4. Double staining of peripheral blood lymphocytes from case No. 6 (×1,600). a: Giemsa staining of atypical lymphocytes. B: TAA-positive cells in the same area as shown in Fig. 4a stained with c143 and FITC-conjugated anti-mouse IgG.

showed a positive reaction for the TAA in the IF test (Fig. 3b).

Case No. 6 showed persistent lymphocytosis (PL) with a CI value of 52.0 at the time of sacrifice. Double staining was performed to determine whether atypical lymphocytes were TAA-positive or not. As shown in Fig. 4a, b, atypical lymphocytes were all TAA-positive. When cow No. 6 was killed for histological observations, medullary hyperplasia in the spleen was observed, but on histological tumor was found in any of the tissues and lymph nodes tested. Case No. 7 showed rumen impaction but no clinical signs of EBL. Since this cow was positive for TAA on its PBL (CI value, 65.1), it was sacrificed for pathological examinations. At necropsy, most external and internal lymph nodes were enlarged. Tumors were also found in the abomasum, lung and heart. Case No. 8 had shown PL up until one year before sacrifice, but the

Table IV. Detection of TAA and Provirus, and Pathological Observations of No. 8 Cow at Necropsy

	Detection of TAA by CDAC test (CI)	Detection of BLV antigen (%)	Presence of tumor		Detection of provirus
			Gross	Histology	
PBL	47.6	21.1			Yes
Cervical Ln	17.5	10.5	No	Yes	No
Popliteal Ln	44.8	ND	No	Yes	Yes
Mesenteric Ln	45.3	6.8	Yes	Yes	Yes
Iliac Ln	33.3	8.7	No	Yes	ND

Abbreviations: Ln, lymph node; CI, cytotoxic index; ND, not done.

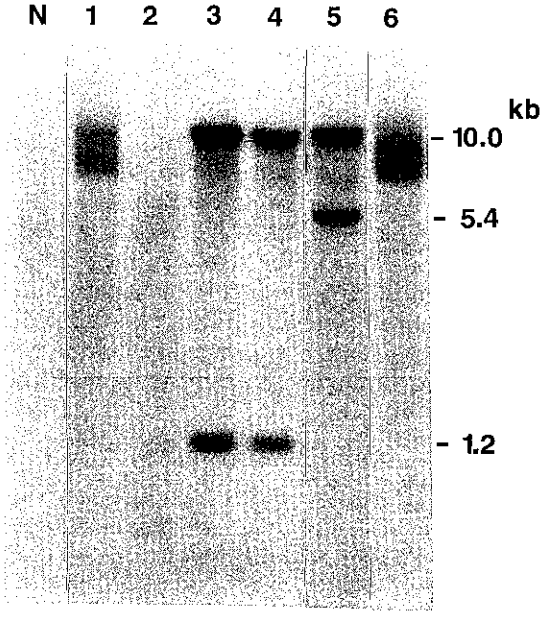

Fig. 5. Integration of BLV proviruses into DNA of case No. 8. Ten micrograms of each DNA was digested with *Eco* RI before electrophoresis, transferred to a nitrocellulose filter and hybridized to the ^{32}P-labeled probe. N: DNA of normal bovine lymph node. 1–4: DNAs from peripheral blood lymphocytes, cervical lymph node, mesenteric lymph node and popliteal lymph node, respectively, of case No. 8. 5: DNA from EBL tumor cells. 6: DNA of peripheral blood lymphocytes obtained from cattle with persistent lymphocytosis.

lymphocyte counts had gradually decreased. At the time of sacrifice, the lymphocyte counts were 7000/μl and the CI value of the PBL was 47.6 (Tables I and IV). At necropsy, a mesenteric lymph node tumor was found macroscopically. Results of the detection of the TAA and provirus, and pathological observations of No. 8 are shown in Table IV. The TAA was detected in popliteal, mesenteric and iliac, but not cervical, lymph nodes. BLV antigen could be detected in cultured lymphocytes from PBL and lymph nodes tested. Proviruses were found on DNA from PBL and mesenteric and popliteal, but not cervical, lymph nodes (Fig. 5). Microscopically, proliferating neoplastic lymphoid cells, mainly lymphoblasts, could be observed in the extending medullary sinus of iliac, popliteal and cervical lymph nodes. In particular, the proliferating neoplastic cells had replaced the normal architecture of the mesenteric lymph node, resulting in complete disorganization.

Discussion

Since it takes a long time for BLV-infected cows to develop EBL, we could not confirm whether or not the TAA-positive animals would develop EBL in the future. However, we were able to sacrifice 8 TAA-positive cattle with no clinical signs of EBL. These 8 animals were necropsied and examined for gross or histological tumors (Table III). Four animals had tumor macroscopically. Three had no tumor but showed follicular hyperplasia positive for the TAA by the IF test, and this was considered to be the initial lesion of the tumor. The remaining animal had no tumors, either grossly or histologically. These results suggest that most of the TAA-positive but

clinically normal animals have tumors and have a higher potential for developing EBL in the future when compared to the BLV-infected but TAA-negative animals.

We have been following up BLV-infected but TAA-negative animals to see if any of them converted to TAA-positive. As shown in Table II, 4 animals, negative for TAA at the time of the first test, converted to positive over a period of 1.5 years. All 4 animals were clinically and hematologically normal even after conversion to TAA-positive. However, at the time of necropsy, 2 animals (Nos. 3 and 4) had tumors macroscopically and 2 showed initial lesions. Since we could not find any tumors histologically in BLV-infected but TAA-negative animals, monitoring of the TAA in PBL might be a good system for finding BLV-infected animals which have a potential to develop leukemia.

The PL is characterized by an increase in the number of PBL,[11] mainly B cells,[12] with or without atypical morphological features but an absence of clinical sings of EBL. The occurrence of EBL in BLV-infected cattle with lymphocytosis is higher than in BLV-infected cattle without lymphocytosis.[13] However, not all cattle with lymphocytosis develop EBL, mainly because a long period of time is necessary for the disease to progress. Previously, we found that most of the cattle with lymphocytosis were positive for the TAA on their PBL.[3, 14] In the present experiment, atypical lymphocytes found in cattle with lymphocytosis were all found to be TAA-positive by the double staining method (Fig. 4). When cows that showed lymphocytosis and were TAA-positive on their PBL were killed for histopathological observations, 2 out of 4 cows had tumors macroscopically at the time of necropsy (Table III). These results suggest that the lymphocytosis is an early stage of EBL and cattle with PL or lymphocytosis may have a greater risk of developing EBL in the future.

It is difficult to determine whether PL or lymphocytosis is merely a reaction stage of the host to BLV before the onset of the malignant period, or is itself a malignant stage. One cow (No. 6), which showed PL, had a high CI value and atypical lymphocytes in its PBL but no gross or histological tumors in any tissue tested. This cow seems to be already in the malignant stage of leukemia, but not lymphosarcoma. It would be interesting to determine the clonality of the proliferating cells by examining the immunoglobulin gene rearrangement pattern to elucidate whether there is a monoclonal proliferation of B-cells.

In the human system, adult T-cell leukemia (ATL) caused by human T-cell leukemia virus type I (HTLV-I) was identified as a clinical entity on the basis of its clinical picture, cell morphology, cell surface marker and geographic distribution.[15] Apart from typical ATL, so-called smoldering ATL has a long course and a tendency for appearance of abnomal cells in the PBL. Since two patients with smoldering ATL, who had skin lesions, developed typical ATL after 5 and 13 years of illness, respectively, it seems possible that smoldering ATL is an early stage of ATL.[16] It is well established that typical ATL develops from anti-HTLV-I antibody-positive healthy individuals, but it is not known which individuals are more likely to develop ATL. Although there is no TAA-like antigen known in the case of ATL, the results of the present experiment may have important implications for the human system; it might be possible to identify the high risk group of ATL if we could find an antigen corresponding to TAA in EBL.

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