FULL PAPER *Pathology*

Immunohistochemical Analysis of Expression Patterns of Tumor Necrosis Factor Receptors on Lymphoma Cells in Enzootic Bovine Leukosis

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ABSTRACT. Tumor necrosis factor-alpha (TNF-α) has been reported to be associated with the progression of lymphoproliferative neoplastic diseases and retroviral infections. Hence we examined immunohistochemically the expression patterns of TNF-receptors (TNF-RI and RII) on lymphoma cells derived from the 29 cases of enzootic bovine leukosis (EBL). Lymphomas obtained in 29 animals with EBL were histopathologically classified into three types: diffuse mixed type (10 cases), diffuse large type (9 cases), and diffuse large cleaved type (10 cases). Immunohistochemically using a monoclonal antibody to a bovine lymphocyte surface antigen, the lymphomas were classified into three phenotypes: B-1a ($CD5^+/CD11b^+$), B-1b ($CD5^-/CD11b^+$) and B-2 (conventional B) ($CD5^-/CD11b^-$). Interestingly, the lymphoma cells in all animals expressed TNF-RII, but not TNF-RI. Although, in EBL, lymphoma cells of which the histopathological and immunological property differs has been formed, the expression patterns of TNF-Rs had the universality in all lymphoma cells. TNF-RII, which induces cell proliferation, was expressed but TNF-RI, which induces cell apoptosis was not expressed on all lymphoma cells, suggesting that TNF-Rs play an important role in the malignant proliferation of B cells and formation of lymphomas in EBL.
KEY WORDS: enzootic bovine leukosis, lymphoma, phenotype, tumor necrosis factor-α, tumor necrosis factor receptor.

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Enzootic bovine leukosis (EBL) is a disease complex of cattle associated with B lymphocytotropic retrovirus, bovine leukemia virus (BLV). Infection by BLV may remain clinically silent as an aleukemic state (AL) or may develop as a persistent lymphocytosis (PL), and more rarely, about 5% of infected animals develop B cell lymphomas in various lymph nodes and organs after a long latent period [3]. Since it takes a long period for the disease to develop, it is believed that BLV and host immune responses are closely related [19]. Many factors relevant to the host immune system have been reported to be associated with processes in the progression of BLV infection, including the activation of cell-mediated immunity [33], major histocompatibility complex (MHC) haplotypes [25], and a mutation in the *p53* gene [47]. In addition, several cytokines have been implicated in this disease progression, such as interleukin (IL)-2, IL-10, IL-12 and interferon (IFN)-γ [21, 22, 29–31, 44]. These reports suggest that cytokines play a major role in BLV infection and the development of clinical symptoms [19]. Furthermore, a recent study of the relationship between tumor necrosis factor-alpha (TNF-α) and BLV infection suggested that TNF-α is a key factor in the disease progression [1, 18, 20, 21, 26, 27].

In BLV infection, cattle develop a sustained nonmalignant polyclonal expansion of $CD5^+$ B cells, also called B-1 cells [8]; this is referred to as PL, with variable progression to lymphoma [2]. It has also been reported that lymphoma cells are classified into three types; B-1a ($CD5^+/CD11b^+$), B-1b ($CD5^-/CD11b^+$) and B-2 ($CD5^-/CD11b^-$) [43].

The importance of TNF-α in the antiviral response and tumor promotion is well established [4, 23, 32, 37, 45]. It has been indicated that TNF-α is involved in the polyclonal expansion of B cells in human retrovirus infections [15, 24], such as chronic lymphocyte leukemia (CLL), an aleukemic form classified according to the presence of B-1 cells [9, 16, 40]. Therefore, in EBL, TNF-α may also be important in other lymphoproliferative diseases such as CLL, because tumors of the B-1 cell type are formed in such diseases. However, the relationship between TNF-α and EBL has not been observed.

In this paper, we report the expressions of TNF receptors (TNF-Rs) on tumor cells obtained from cattle with EBL, discuss the relationship among TNF-Rs expression, phenotype of tumor cells and cellular morphology, and attempt to clarify the pathogenesis of EBL.

MATERIALS AND METHODS

Experimental animals: Twenty-nine animals with EBL diagnosed on the basis of clinicopathological findings at necropsy were used in this experiment. These animals were obtained from 1991 to 2003 at the Department of Veterinary Pathology, Faculty of Agriculture, Iwate University. Details of the animals are listed in Table 1.

Five BLV-uninfected healthy animals from an abattoir were used as controls for immunohistochemical examination of TNF-Rs.

Hematologic examination: Blood samples collected from the jugular vein were used for hematologic examination in all cases. The total number of leukocytes was counted with

Table 1. Clinical and hematological findings of enzootic bovine leukosis

Animal No.	Protocol No.	Breed[a)]	Sex[b)]	Age[c)]	ID[d)] gp, p	Hematological findings		
						WBC[e)]	Lymphocytes[f)]	AtMC[g)]
1	2654	HF	F	4y	+, –	71,400	79	6
2	2662	HF	F	7y	+, +	9,800	46	20
3	2663	HF	F	5y	+, –	171,000	10	88
4	2673	HF	F	5y	+, –	24,800	31	46
5	2682	HF	F	4y	+, –	9,000	45	16
6	2686	JB	F	7y	+, –	24,600	70	29
7	2726	F1	F	9y	+, +	38,300	21	36
8	2732	HF	F	7y	+, +	8,400	65	8
9	2737	JB	F	6y	+, +	15,000	18.5	0
10	2738	JB	F	10y	+, +	162,000	18.5	54.5
11	2744	JB	F	5.5y	+, +	10,540	0	95
12	2745	JB	F	4.9y	+, +	33,700	47.5	7
13	2746	JB	F	5y	+, –	20,700	43.5	4.5
14	2684	HF	F	6y	+, –	75,500	7	89
15	2687	HF	F	10y	+, +	40,500	28	70
16	2747	JB	F	9y	+, –	8,600	10	15
17	2752	JB	F	10y	+, +	18,300	0	98
18	2197	HF	F	4y	+, –	11,800	43	22
19	2377	HF	F	10y	+, –	13,800	21	8
20	2406	HF	F	7y	+, –	24,100	60.7	15.9
21	2648	JB	M	6y	+, +	18,400	40	37
22	2660	JB	F	10y	+, –	13,100	20	57
23	2681	JB	F	13y	+, –	7,300	29	32
24	2685	JB	F	11y	+, +	39,700	33.5	59.5
25	2739	JB	F	7y	+, +	14,700	25.5	3.5
26	2750	HF	F	7y	+, +	12,000	24	1
27	2513	JB	F	7y	+, +	133,600	83	16
28	2193	JS	F	9y	+, –	6,000	39.8	23
29	2614	JB	F	5y	+, –	6,900	68	1

Notes a) JB: Japanese black, JS: Japanese shorthorn, HF: Holstein-Friesian, F1: First filial of Japanese black/Holstein-Friesian.
b) M: Male, F: Female.
c) y: Years.
d) ID: Immunodiffusion test, gp, p: Serum antibodies to glycoprotein and protein antigens of BLV.
e) WBC: Number of white blood cells/mm^3.
f) Lymphocytes: Percentage of lymphocytes (%).
g) AtMC: Percentage of atypical mononuclear cells (%).

an automatic blood cell counter (Sysmex cc-110, Tokyo, Japan). The percentage of white blood cells on blood smears subjected to May-Giemsa staining. The presence of serum antibodies to gp51 and p24 of BLV was determined by the agar gel immunodiffusion test.

Preparation of samples and histopathological examination: All the animals were euthanized by bleeding and necropsied. After a gross observation, tumor tissues and lymphnodes of BLV-uninfected healthy animals were fixed in 10% neutral buffered formalin and embedded in paraffin. Paraffin sections were stained with hematoxylin and eosin (HE). Blocks of tumor tissues and lymphnodes of BLV-uninfected healthy animals were also frozen rapidly by immersion in liquid nitrogen and stored at –80°C until cryostat sectioning for immunohistological examination.

Immunohistological examinations with monoclonal antibodies (MoAbs): MoAbs against bovine differentiation antigen for lymphocytes used in immunohistochemical staining were as follows: MM1A (anti-BoCD3) (VMRD, Pullman, WA), MM10A (anti-BoCD11b) (VMRD), CACT138 (anti-BoCD4) (VMRD), CACT80C (anti-BoCD8) (VMRD), TH14B (anti-BoMHC class II) (VMRD), LCT27A (anti-B cell) (VMRD), PIg45A (anti-sIgM) (VMRD), BIg501E (anti-γ light chain) (VMRD), BIg623A (ani-IgG_2) (VMRD) and CC17 (anti-BoCD5) (Serotec, UK). All these MoAbs were used at 15 μg ml^{-1}. Specimens were cut by cryostat sectioning and the obtained thin sections that were stained by the dextran polymer method using a DAKO EnVision^{+TM} kit (goat anti-mouse immunoglobulins conjugated to peroxidase labeled-dextran polymer) (Dako Cytomation, Via Real Carpinteria, CA). Peroxidase activity was determined using 3, 3'-diaminobenzidine tetrahydrochloride.

Analysis of TNF-Rs expression: To identify cells expressing TNF-Rs, the indirect fluorescent antibody (IFA) technique was performed using the following antibodies: H-271 (rabbit anti-human TNF-RI antibody, Santa Cruz Biotechnology, Santa Cruz, CA) and H-202 (rabbit anti-human TNF-RII antibody, Santa Cruz Biotechnology). Specificity

of the antibodies against TNF-Rs (H-271, H-202) was confirmed by flow cytometry analysis using concanavalin A (Con A) stimulated bovine peripheral blood mononuclear cells (PBMCs). Thin sections obtained by cryostat sectioning were incubated with the appropriate antibody at the optimal concentration for 60 min at 4°C, washed three times in Tris-buffered saline (TBS), and then reacted with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (H+L) $(Fab')_2$ (Zymed Laboratories Inc., South San Francisco, CA) for 60 min at 4°C. After the staining, samples were immediately observed under a fluorescence microscope.

Evaluation of TNF-Rs expression and statistical analysis: Four random FITC and transmitted light images were captured by a digital camera. These images were stored in a personal computer. FITC and transmitted light images were obtained using Adobe Photoshop software (Version 6, Adobe Systems). Then the percentage of TNF-R-positive cells in 200 cells was determined. These data were analyzed statistically by the Student's *t*-test.

RESULTS

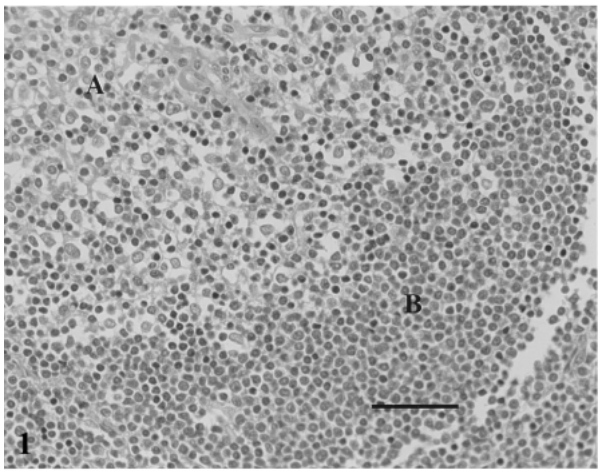
Hematologic examination: Hematologic findings are shown in Table 1. According to the criteria of Bendixen [2], nine animals (Case Nos. 5, 9, 16, 19, 23, 25, 26, 28 and 29) were diagnosed as having aleukemic leukosis and the rest were diagnosed as having leukemic leukosis. All the animals with EBL cases were positive for the BLV antigen as determined by agar gel immunodiffusion test.

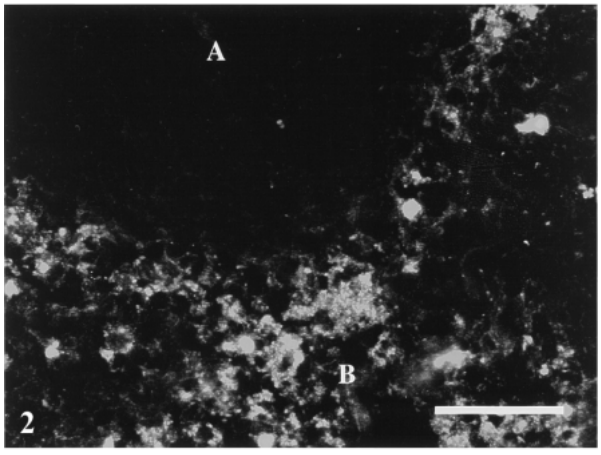
Histopathologic examination: The collected tumors were classified into three types according to the morphological features of neoplastic cells. In all animals, the lymphomas were of high grade, because tumor cells infiltrated many organs and diffusely proliferated. The tumor of the diffuse mixed cell type was composed of small nucleus-cleaved lymphocytes and large immunoblastic lymphocytes. Mitotic cells were extremely rare (Fig. 4). The tumor of the diffuse large cell type was composed of large immunoblastic lymphocytes. Nuclei were round. Mitotic cells were few (Fig. 6). The tumor of the diffuse large cleaved cell type was composed of large immunoblastic lymphocytes. Nuclei were cleaved. Mitotic cells were few (Fig. 8). The histological types of tumors in the animals are listed in Table 2. The mixed type, large type, and large cleaved type were observed in 10, 9, and 10 animals, respectively.

The lymphnodes of BLV-uninfected healthy animals were observed germinal center and paracortex regions composed of small lymphocytes (Fig. 1).

Immunohistochemical examination of tumor cells: The immunohistochemical findings of tumor cells are shown in Table 2.

All tumor cells were negative for CD3, CD4 and CD8. The animals showed some lymphocytes positive for these markers. These cells were normal T cells that infiltrated neoplastic tissues. However, all of the tumor samples were positive for B cell marker (LCT27A); tumors that were B





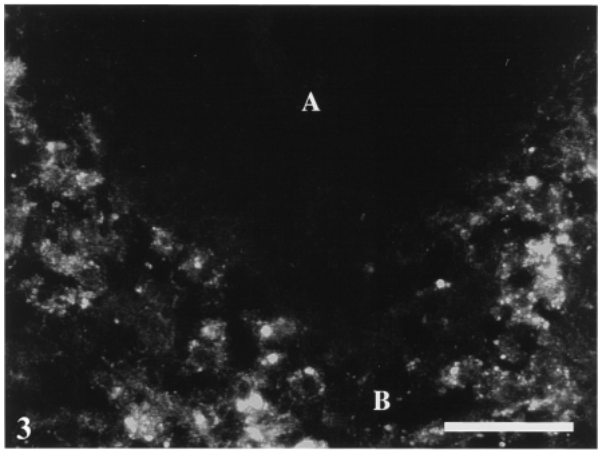


Fig. 1. Mesenteric lymphnode of BLV-unifected healthy cows. Germinal center (A) and paracortex region (B) were observed. Hematoxylin and eosin (HE) stain. Scale bar = 50 μm.

Fig. 2. Mesenteric lymphnode of BLV-unifected healthy cows. Lymphocytes of paracortex region were expressed TNF-RI (B) (green signal by FITC). (A) Germinal center. Scale bar = 50 μm.

Fig. 3. Mesenteric lymphnode of BLV-unifected healthy cows. Lymphocytes of paracortex region were expressed TNF-RII (B) (green signal by FITC). (A) Germinal center. Scale bar = 50 μm.

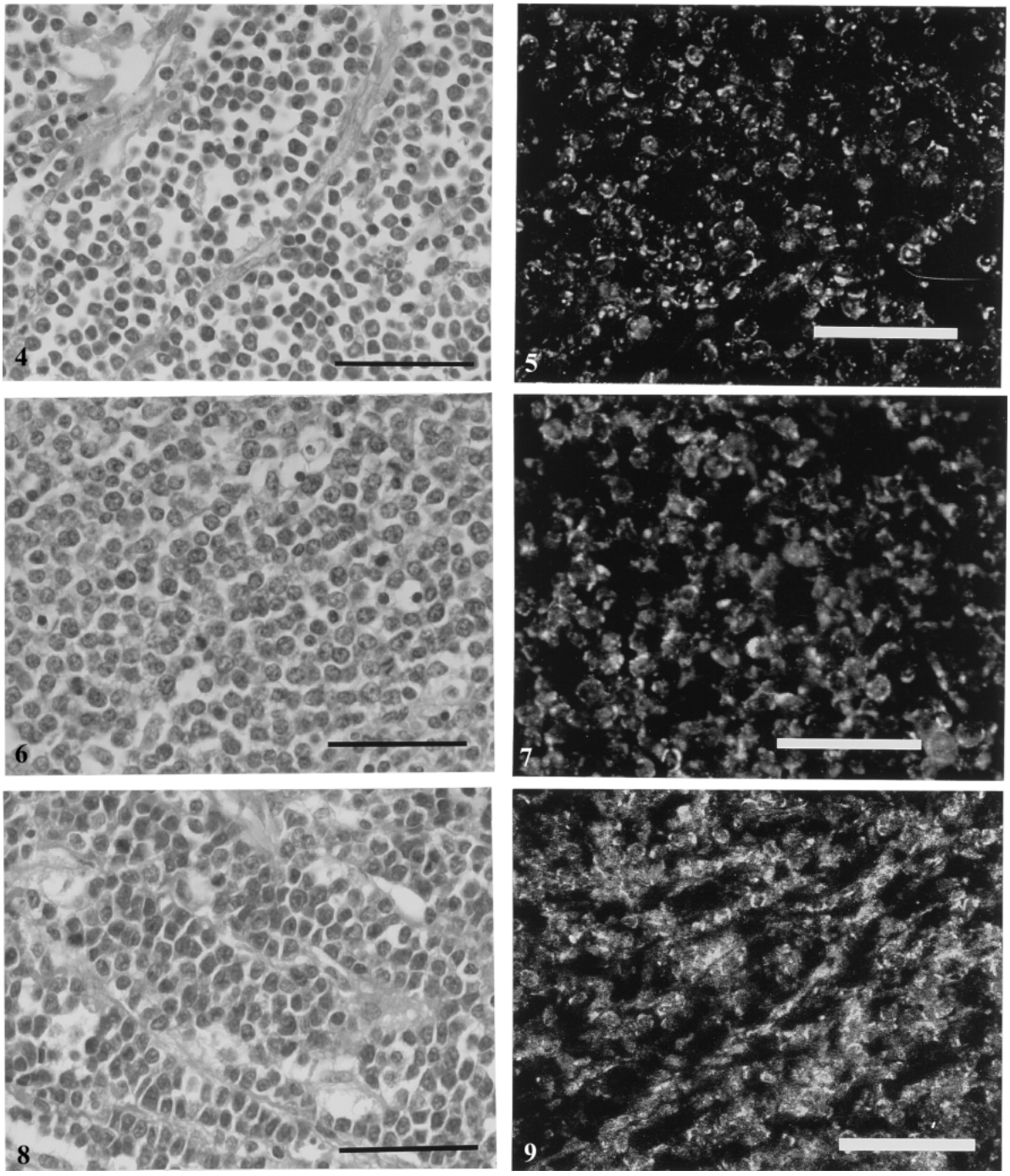


Fig. 4. Tumor tissue from animal No. 26. Diffuse mixed cell type. Tumor cells are small and large in size and diffusely proliferated. HE stain. Scale bar = 50 μm.

Fig. 5. Immunofluorescence staining test on a frozen section of tumor tissue (animal No. 26). All of tumor cells are TNF-RII positive (green signal by FITC). Scale bar = 50 μm.

Fig. 6. Tumor tissue from animal No. 19. Diffuse large cell type. Tumor cells are large in size and diffusely proliferated. HE stain. Scale bar = 50 μm.

Fig. 7. Immunofluorescence staining test on a frozen section of tumor tissue (animal No. 19). All of tumor cells are TNF-RII positive (green signal by FITC). Scale bar = 50 μm.

Fig. 8. Tumor tissue from animal No. 12. Diffuse large cleaved cell type. Tumor cells are large in size and have cleaved nucleus. The cells are diffusely proliferated. HE stain. Scale bar = 50 μm.

Fig. 9. Immunofluorescence staining test on a frozen section of tumor tissue (animal No. 12). All of tumor cells are TNF-RII positive (green signal by FITC). Scale bar = 50 μm.

Table 2. Immunohistological and histological findings of tumor cells

Animal No.	Differentiation antigen of bovine lymphocyte										Histological type
	BoCD5	BoCD11b	sIgM	IgG_2	B cell	λ light chain	MHC class II	BoCD3	BoCD4	BoCD8	
1	+[a)]	±[b)]	±	–[c)]	+	±	+	–	–	–	large
2	+	+	+	–	+	±	+	–	–	–	mixed
3	+	+	–	–	+	±	+	–	–	–	mixed
4	+	+	–	–	+	+	+	–	–	–	mixed
5	+	+	+	+	+	+	+	–	–	–	large cleaved
6	+	+	–	–	+	+	±	–	–	–	large
7	+	+	±	–	+	+	+	–	–	–	mixed
8	+	+	–	–	+	+	+	–	–	–	mixed
9	+	+	±	–	+	+	+	–	–	–	mixed
10	+	+	–	–	+	–	+	–	–	–	mixed
11	+	+	+	–	+	+	+	–	–	–	large
12	+	+	–	–	+	±	+	–	–	–	large cleaved
13	+	+	–	–	+	+	+	–	–	–	large cleaved
14	–	+	–	–	+	+	±	–	–	–	large
15	–	+	+	–	+	+	+	–	–	–	large
16	–	±	+	–	+	+	+	–	–	–	large cleaved
17	–	+	–	–	+	+	+	–	–	–	large cleaved
18	–	+	–	–	+	+	–	–	–	–	large cleaved
19	–	+	+	±	+	±	+	–	–	–	large
20	–	+	+	+	+	+	+	–	–	–	large
21	–	+	±	–	+	+	+	–	–	–	mixed
22	–	–	–	–	+	+	+	–	–	–	large
23	–	–	–	–	+	+	+	–	–	–	large cleaved
24	–	–	–	–	+	+	+	–	–	–	large cleaved
25	–	–	–	–	+	+	±	–	–	–	large cleaved
26	–	–	–	–	+	–	±	–	–	–	mixed
27	–	–	–	±	+	+	+	–	–	–	large
28	–	–	±	–	+	±	+	–	–	–	large cleaved
29	–	–	–	–	+	–	+	–	–	–	large

Notes a) –: negative, ±: diffusely positive, +: positive.

cells in origin confirmed by LCT27A. MHC class II was expressed in almost all tumor cells. The majority of tumor cells showed positive reactions to CD5 and CD11b in Nos. 1–13. CD11b was expressed, but not CD5 in Nos. 14–21. CD5 and CD11b were not detected in Nos. 22–29.

The sIgM was present in 12 animals (Nos. 1, 2, 5, 7, 9, 11, 15, 16, 19–21 and 28) , and IgG_2 was present in 4 animals (Nos. 5, 19, 20 and 27). All animals except Nos. 10, 26 and 29 were positive for the γ light chain.

It was possible to classify the tumor cells into three phenotypes from the expression patterns of CD5 and CD11b: $CD5^+/CD11b^+$ cells called B-1a were observed in 13 animals (Nos. 1–13), $CD5^-/CD11b^+$ cells called B-1b in 8 animals (Nos. 14–21), $CD5^-/CD11b^-$ cells called B-2 (conventional B) in 8 animals (Nos. 22–29). Furthermore, together with the findings of sIgM, MHC class II, IgG_2 and γ light chain, tumor cells were classified into 13 phenotypes.

Examination of TNF-Rs expression on tumor cells: On ConA-stimulated PBMC from normal cow, there were no different expressions between TNF-RI and TNF-RII (data not shown). Both TNF-RI and TNF-RII were expressed on lymphocytes of paracortex regions in lymphnode of BLV-uninfected healthy cows (Figs. 2, 3).

The results of TNF-Rs expression analysis are summarized in Table 3 for the three types in immunohistological phenotypes of tumor cells. All tumor cells expressed TNF-RII (Figs. 5, 7, 9). However, TNF-RI was not expressed on tumor cells in all immunohistological phenotypes (Table 3).

There was no statistically significant difference in the number of tumor cells that expressed TNF-RII between the three immunohistological phenotypes. These results were the same as those for 29 animals and three histological types. Therefore, all tumor cells in EBL has uniformly expressed the TNF-RII, but not TNF-RI.

DISCUSSION

EBL indicates the malignant monoclonal proliferation of B cells. However, these neoplastic B cells are classified into different phenotypes by immunohistochemical analysis using a monoclonal antibody to leukocyte differentiation antigen [6, 7, 13, 42, 43, 46]. Furthermore, tumor cells in EBL were classified into three or more types on the basis of histological findings [17, 39]. In this study, the tumor cells were classified into three types on the basis of the presence or absence of CD5 and CD11b. The CD5 that is typed in humans and mouse mature T cell membrane and CD11b marker that is seen in monocytes and granular cells as an adhesion molecule have been found at the surface of partial B lymphocytes [5, 14]. They have been also found in B cell

Table 3. Expression of TNF-Rs

Phenotype: B-1a cell lymphoma in EBL

Animal No.	Number of TNF-Rs- positive cells (Mean ± SD)	
	TNF-RII	TNF-RI
1	187.50 ± 11.21	0
2	185.00 ± 4.83	0
3	189.50 ± 6.56	0
4	186.25 ± 4.11	0
5	196.00 ± 2.94	0
6	191.50 ± 6.45	0
7	190.00 ± 6.58	0
8	197.00 ± 1.83	0
9	187.25 ± 4.03	0
10	192.75 ± 4.79	0
11	194.75 ± 2.06	0
12	186.00 ± 13.93	0
13	192.50 ± 5.51	0
Mean ± SD	190.46 ± 3.97	0

Phenotype: B-1b cell lymphoma in EBL

Animal No.	Number of TNF-Rs-positive cells (Mean ± SD)	
	TNF-RII	TNF-RI
14	192.50 ± 3.70	0
15	199.25 ± 1.50	0
16	197.50 ± 3.00	0
17	193.25 ± 1.71	0
18	195.25 ± 3.86	0
19	157.50 ± 3.86	0
20	160.25 ± 11.79	0
21	167.00 ± 12.44	0
Mean ± SD	182.81 ± 17.90	0

Phenotype: B-2 cell lymphoma in EBL

Animal No.	Number of TNF-Rs-positive cell (Mean ± SD)	
	TNF-RII	TNF-RI
22	187.00 ± 8.49	0
23	188.50 ± 12.82	0
24	187.50 ± 7.94	0
25	187.00 ± 5.83	0
26	194.75 ± 1.71	0
27	181.75 ± 9.00	0
28	191.50 ± 5.92	0
29	174.00 ± 14.35	0
Mean ± SD	186.50 ± 6.29	0

chronic lymphocyte leukemia in humans [16]. The same cells have also been observed in normal B lymphocytes and tumor-like B cells infected with BLV [8, 28]. In addition, they were subdivided into 12 types, when IgG_2, sIgM and γ light chain were also taken into consideration and into three types on the basis of histopathological findings. Wu *et al.* [43] and Yin *et al.* [46] have demonstrated that tumor cells in BLV-induced lymphomas present the phenotypic and histological diversity. This is why, the pathogenesis of BLV-induced tumor cells are not required of single B cell origin. Tumor cells were formed as a result of the effect of various modulators such as the selection of BLV-infected cells by cellular immunity [19], activation of proliferative responses and malignant transformation by TNF-α and its receptors [18, 21, 27].

To confirm the hypothesis, we have investigated the expression of TNF-Rs on lymphoma cells from animals with BLV-induced EBL. Interestingly, the lymphoma cells of all animals expressed the TNF-RII, but not TNF-RI. Although, the histopathological and immunological properties of neoplastic cells were variable among EBL cattle, the expression patterns of TNF-Rs had the universality in all lymphoma cells. TNF-α is a pleiotropic cytokine, which is involved in diverse biological processes including immune and inflammatory reactions. Furthermore, TNF-α plays a critical role in the elimination of some infectious agents [21]. On the other hand, its function may promote disease progression [11, 12]. TNF-α activity is mediated by two functionally different cell surface receptors, TNF-RI and TNF-RII [34]. TNF-RI and -RII are coexpressed in several different tissues. In the case of human B cells, most resting peripheral blood B cells express low levels of TNF-RII, which is markedly up-regulated upon stimulation with a B cell mitogen. In contrast, the expression level of TNF-RI was low on activated B cells [40]. Most biologic responses of TNF-α, such as the induction and suppression of apoptosis, are considered to be mediated by these two different receptors [10, 11, 35, 36, 41]. Some investigations have revealed the relationships between TNF-α or its receptors and B-cell malignant transformations such as in the case of chronic lymphocytic leukemia [38, 41], Epstein-Barr (EB) virus-transformed B cells, and Burkitt lymphoma [11]. In these lymphomas, TNF-α is involved in the proliferation of leukemic cells through its binding to TNF-RII. In this study, EBL tumor cells expressed TNF-RII, but not TNF-RI. An other study has indicated that TNF-RI is not expressed on KU-1, which is a bovine B cell line transformed by BLV [18]. These results suggest that TNF-RII carries out important functions in BLV-induced leukemogenesis like other lymphomas.

We consider that TNF-Rs are important for the formation of lymphomas in EBL. However, in future studies, the observations of TNF-α expression in neoplastic tissues in EBL and TNF-Rs expression in AL and PL will be necessary to clarify the process by which EBL arises from BLV infection.

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