Pathological Findings of Two Types of Lymphoid Malignancy in Sheep Inoculated with Bovine Leukemia Virus

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(Received 5 December 1988/Accepted 16 April 1989)

ABSTRACT. Different types of lymphoid malignancy were observed in two sheep inoculated with BLV-containing materials. Sheep 1 showed severe leukemic change in the peripheral blood and splenomegaly but lymphosarcoma in the lymph nodes was absent. Sheep 2 had lymphosarcoma in the lymph nodes and various organs. Neoplastic cells had B-cell marker in both cases and a few neoplastic cells contained intracytoplasmic IgM in sheep 2. It was presumed that B-cells might be transformed into neoplastic cells on the way of their differentiation. Some of neoplastic cells might have ability of immunoglobulin-production in sheep 2.—**KEY WORDS:** bovine leukemia virus, bovine leukosis, intracytoplasmic immunoglobulin, ovine lymphosarcoma, surface immunoglobulin.

Jpn. J. Vet. Sci. 51(4): 749–756, 1989

The development of lymphosarcoma in sheep after neonatal injection of whole blood from cattle showing the persistent lymphocytosis (PL) of enzootic bovine leukemia (EBL) was reported by Wittmann *et al.* [18]. After it was shown that a c-type virus (bovine leukemia virus-BLV) was associated with EBL [7], many attempts to induce lymphosarcoma in sheep by inoculation of BLV-containing materials have been done [3, 5, 8, 10, 11]. It was confirmed that BLV was infectious and leukemogenic for sheep [10]. The characteristic lesion in sheep infected with BLV appeared in the lymph nodes, sometimes with sarcomatous lesion of various organs [11].

In order to obtain more definitive evidence for the leukemogenicity of BLV, we injected BLV-containing materials into sheep by various routes. Two cases showed different types of lymphoid malignancy, respectively. One case showed severe leukemic change in peripheral blood and massive splenomegaly in spite of absence of lymph node-neoplasia and another case showed

lymphosarcoma in lymph nodes accompanied with neoplastic lesion of various organs. Histological features and immunological observations of both cases were described.

MATERIALS AND METHODS

Animals: Two sheep were tested and found negative for antibodies to glycoprotein (gp) and inner protein (p) of BLV using agar gel immunodiffusion test [6] before experiment. Sheep 1 was inoculated intraperitoneally at 3 months of age with 1 ml of cell-free supernatant which was concentrated 100 times after 48 hr-culture of lymphocytes from a cow with EBL. Sheep 2 was inoculated intracutaneously at 13 months of age with 0.05 ml of whole blood from sheep which survived for 12 months after inoculation of cell-free BLV preparation obtained from fetal lamb kidney cells persistently infected with BLV [17].

Hematological and virological examinations: The number of peripheral leukocytes

was counted by a Coulter Counter Model ZF (Coulter Electronics Inc., U.S.A.) and percentage of differential white blood cells was enumerated on blood smears. Serum samples were collected periodically and were tested for the presence of antibodies to gp and p of BLV using agar gel immunodiffusion test [6]. Lymphocytes collected from peripheral blood and tumor tissues were subjected to syncytia infectivity assay using primary passage of bovine embryonic spleen cells [2] and phytohemagglutinin (PHA) stimulated culture for the detection of virus particles under an electron microscope (EM).

Pathological examinations: At necropsy tissue samples were fixed in 10% buffered formalin solution and processed for paraffin sectioning by routine methods. Thin sections were stained with hematoxylin and eosin and silver impregnation. For electron microscopy, the buffy coat of peripheral blood, pellets of PHA-treated lymphocytes and small blocks of neoplastic tissues were fixed in 2.5% glutaraldehyde solution, post-fixed in 1% osmium tetroxide solution and embedded in Epon 812. Ultrathin sections were stained with uranyl-acetate and lead-citrate.

Immunological examinations: For detection of surface immunoglobulin (sIg), mononuclear cells were isolated from peripheral blood or tumor tissues as described previously [14]. The cells from sheep 1 were obtained from peripheral blood and stored with the medium containing 10% dimethylsulfoxide in liquid nitrogen. The ratio of viable cells were approximately 90%. The cells from sheep 2 was isolated from fresh tumor tissues and peripheral blood. These cells were washed in PBS, adjusted to 1×10^6 cells per ml of suspension, mixed with 50 μ l of 1:20 dilution of fluoresceinated anti-sheep IgG rabbit serum (Cappel Worthington, U.S.A.) and incubated for 30 min in ice water bath. These

cells were washed and 200 live cells were counted under a fluorescent microscope.

Detection of cytoplasmic immunoglobulin (cIg) was performed by using biotin-streptavidin system (BioGenex Lab., U.S.A.). Paraffin sections were blocked with 3% H_2O_2 before incubation. The sections were incubated for 30 min at 1:1000 dilution of rabbit anti-sheep IgM (μ -chain specific, Cappel Worthington, U.S.A.) or IgG (Fc specific, EY Lab, U.S.A.). After washing the sections were incubated in biotin-conbined anti-rabbit goat serum for 20 min and washed again. The sections were exposed to a peroxidase-conjugated streptavidin for 20 min. Peroxidase activity on sections was detected with a standard diaminobenzidine solution.

RESULTS

Hematological and virological observations: A marked increase to leukemic levels of circulating lymphocytes was noted in both sheep. The number of lymphocytes was 760,000 cells per μ l of blood in sheep 1 at 13 months after inoculation and 88,000 cells per μ l of blood in sheep 2 at 29 months after inoculation. On blood smears white blood cells were mostly occupied by atypical lymphoid cells. Under EM, these cells had round or oval shaped nuclei with scant heterochromatin and small amounts of mitochondria, rough and smooth-surfaced endoplasmic reticulum. Ribosomes were rather abundant (Fig. 1-A).

In two sheep gp antibody to BLV was detected within one month after inoculation and p antibody was within 6 months after inoculation. These antibodies persisted until the last evaluation at necropsy. Lymphocytes collected from sheep 1 formed syncytia on cultivated primary bovine embryonic spleen cells as early as 3 months after inoculation. A c-type virus particle was demonstrated in PHA-treated lymphocytes

Fig. 1-A. Electron microscopy of peripheral blood in sheep 1. Neoplastic cells contained round or oval nuclei and small amounts of mitochondria and endoplasmic reticulum. Bar: $10\mu\text{m}$. $\times 1250$.

Fig. 1-B. C-type virus from phytohemagglutinin-stimulated lymphocyte. Bar: $0.1\mu\text{m}$. $\times 60,000$.

from sheep 1 under EM (Fig. 1-B).

Macroscopical observations: Sheep 1 was sacrificed at 13 months after inoculation. The spleen was greatly enlarged 5 times of normal size. Lymphadenopathy and neoplastic lesion of other organs were conspicuously absent. Sheep 2 was sacrificed at 29 months after inoculation. Almost all lymph nodes were markedly enlarged. Tumorous enlargement of the spleen was milder than that of sheep 1 and it measured up to one and a half times of normal size. There was slight hepatomegaly.

Microscopical observations: In sheep 1 splenomegaly was dependent on extensive proliferation of neoplastic cells in red pulp as well as in sinuses (Fig. 2). The preservation of islands of atrophic white pulp was obscurely observed in the cellular proliferation. Most of the lymph nodes examined were not involved by lymphoid neoplasia.

They showed atrophy of lymphatic tissue and occasional infiltration of macrophages in sinuses. While a moderate number of neoplastic cells packed in sinuses of a few lymph nodes (Fig. 3). There was hyperplasia of lymphatic follicles in the prefemoral node and plasma cell infiltration in medullary cords of the popliteal node. Moderate infiltration of neoplastic cells was present in the heart and uterus. Intravascular infiltration of neoplastic cells was present in various organs. There was no neoplastic lesion in the bone marrow.

In sheep 2 the spleen had intrasinusoidal accumulation of neoplastic cells without accompanying destruction of parenchyma (Fig. 4). Most of the lymph nodes were occupied by extensive proliferation of neoplastic cells and the normal structure was no longer recognized (Fig. 5). Lymphatic vessels and sinuses were packed with neoplastic

Fig. 2. Neoplastic proliferation in spleen of sheep 1. Splenic parenchyma is replaced by neoplastic cells. Hematoxylin and eosin stain (HE). $\times 250$.

Fig. 3. Neoplastic infiltration in rumenal lymph node of sheep 1. Sinuses are packed with neoplastic cells. HE. $\times 250$.

Fig. 4. Neoplastic infiltration in spleen of sheep 2. Sinuses are packed with numerous neoplastic cells. HE. $\times 250$.

Fig. 5. Neoplastic infiltration in submandibular lymph node of sheep 2. Normal lymphatic tissue is no longer seen. HE. $\times 250$.

cells. In the liver a large number of neoplastic cells were seen in almost all portal blood vessels and sinusoids. Neoplastic infiltration was observed in interstitial tissue of the kidneys and pancreas, intramuscular connective tissue of the heart and submucosal tissue of the esophagus and abomasum. Various organs showed slight to moderate intravascular infiltration of neoplastic cells.

Neoplastic cells of sheep 1 were slightly smaller than those of sheep 2 and accorded with small to medium cell type in the classification of lymphoma study group (LSG) [13]. They contained round or oval nuclei, sometimes with small indentations, and scant cytoplasm with small amounts of mitochondria, rough and smooth surfaced endoplasmic reticulum and large amounts of

Fig. 6. Electron microscopy of neoplastic cells in rumenal lymph node of sheep 1. Nuclei are round or oval in shape sometimes with irregular indentations. Bar: $2\mu\text{m}$. $\times 4,000$.

Fig. 7. Electron microscopy of neoplastic cells in prescapular lymph node of sheep 2. Nuclei are irregular in shape. Bar: $2\mu\text{m}$. $\times 3,200$.

Fig. 8. Surface immunoglobulin assay in prescapular lymph node of sheep 2. Most of neoplastic cells show positive reaction. Fluorescent microscopy. $\times 800$.

Fig. 9. Intracytoplasmic IgM positive cells in submandibular lymph node of sheep 2. Biotin-streptavidin system. $\times 400$.

ribosomes under EM (Fig. 6). Neoplastic cells of sheep 2 were more atypical than those of sheep 1 and regarded as medium cell type in LSG classification. The nuclei were commonly irregular in shape and moderate amounts of ribosomes were present in cytoplasm (Fig. 7).

Immunological observations: The percentage of sIg positive cells (Fig. 8) of peripheral blood lymphocytes from sheep 1 and 2 was 90.0% and 90.8%, respectively. Lymphocyte preparation from neoplastic lymph nodes of sheep 2 contained 97.8% sIg positive cells. In the neoplastic cells of

spleen and lymph nodes of sheep 1, intracytoplasmic IgM and IgG were not detected by biotin-streptavidin system. A few intracytoplasmic IgM-positive cells were detected among neoplastic cells in the lymph nodes of sheep 2 (Fig. 9).

DISCUSSION

In the present cases, two sheep inoculated intraperitoneally and intracutaneously, respectively, with BLV-containing materials developed an antibody response in the same period after inoculation and BLV was demonstrated from one of them by syncytia-induction assay and under EM. These findings strongly suggested the neoplastic lesions in these cases resulted from neoplastic transformation by leukemogenicity of BLV as described in many reports [3, 5, 10, 11]. Unfortunately, owing to a small number of cases showing lymphoid malignancy it was not possible to speculate from the present study that a shorter latent period in sheep 1 inoculated intraperitoneally might depend upon difference in the route of inoculation and preparation of inoculum.

The similarity of lymphosarcoma involving certain lymph nodes, heart, abomasum, uterus etc. in cattle [19] was noted in sheep [11]. At the present study, however, sheep 1 showed severe leukemic change in peripheral blood with marked splenomegaly but lymphosarcoma was absent in the lymph nodes. Johnston *et al.* [4] described that neoplastic lesion of lymph nodes was absent in 3 naturally occurring lymphoma of sheep, which had only lesion in the kidneys. In prolymphocytic leukemia of man severe leukemic change in peripheral blood, massive splenomegaly and lympho-proliferation in the bone marrow were observed but lymphadenopathy was absent [12]. The neoplastic lesion of sheep 1 was not accordant with the report of the naturally occurring lymphoma in sheep but it was similar to

prolymphocytic leukemia in man with exception of absence of neoplastic lesion in the bone marrow. Judging from our observations and a report that neoplastic involvement of the bone marrow was scarcely recognized in EBL [19], the neoplastic cells in sheep inoculated with BLV might have a nature which was non-proliferation in the bone marrow. On the other hand, sheep 2 had lymphosarcoma in the kidneys, pancreas, abomasum and esophagus as well as in the lymph nodes. This distribution of the lesions was similar to that of lymphosarcoma in EBL [19]. Different types of neoplastic lesion between 2 cases might be dependent on site of initial transformation and proliferation of neoplastic cells. It was presumed that the spleen in sheep 1 and the lymph nodes in sheep 2 might have adequate environment for initial proliferation of neoplastic cells, respectively.

Hyperplastic lymphatic follicles which were observed in a non-neoplastic lymph node of sheep 1 were similar to findings described as initial lesion of EBL [9]. This suggested that BLV might stimulate B-cells in lymphatic follicles during preleukotic stage. An increase of plasma-cells in a lymph node of the same sheep might depend on acceleration of differentiation from B-cells.

Urbaneck *et al.* [16] considered that neoplastic cells of sheep inoculated with blood from cattle with PL might be derived from thymus. By using acid phosphatase and T-cell specific naphthylacetate esterase staining, T-cell lymphoblastoma was diagnosed in two sheep inoculated with BLV [8]. On the contrary, Takashima *et al.* [15] demonstrated B-cells in lymphoma of BLV-infected sheep by sIg assay. Naturally occurring ovine lymphomas were classified into T-cell-, B-cell-, double marker cell- and null cell-derived tumors [1].

In the present cases, the neoplastic cells had cytomorphologically slight differences

between two cases but both of them possessed sIg as B-cell marker. Therefore it was clear that neoplastic transformation occurred on the way of differentiation of B-cells. Some of neoplastic cells might have ability of production of intracytoplasmic immunoglobulin in sheep 2. More detailed studies should be carried out on determination for the stage of B-cell differentiation in which neoplastic transformation might occur.

ACKNOWLEDGEMENTS. The authors wish to thank Messrs. M. Ito and Y. Ando for preparation of photographs.

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要 約

牛白血病ウイルス接種羊に発現した2型のリンパ腫病変の病理学的観察：石野清之・松田 泉¹⁾・山本春弥²⁾・吉野知男・泉対 博・水野喜夫・甲野雄次（農林水産省家畜衛生試験場，¹⁾同省農業環境技術研究所，²⁾山口大学農学部家畜病理学教室）——牛白血病ウイルスを接種された羊の経過観察中に2頭が発症し，それぞれ異なった腫瘍病変を示した．第1例はリンパ節病変を欠き，巨脾と高度の白血性変化を示し，第2例はリンパ節と他の組織に高度なリンパ肉腫性病変を示した．両例の腫瘍細胞はB細胞のマーカーを有し，第2例では少数の細胞の細胞質内にIgMが見られた．いずれの腫瘍細胞もBリンパ球の分化過程で腫瘍化したものと考えられ，第2例では一部の細胞が免疫グロブリン産生能力を獲得したと類推された．