Partial Purification of a Common Antigen in Bovine Lymphoma and Its Use in a Lymphocyte Blastogenesis Assay[1]

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ABSTRACT

An antigen was isolated from tumor cells derived from cases of bovine lymphoma which caused the in vitro blastogenesis of lymphocytes from nine of 13 (69%) adult cattle with lymphoma. Lymphocytes derived from five of 122 (4%) normal adult cattle also underwent blastogenesis while lymphocytes from three cases of the sporadic form of lymphoma did not respond. Blastogenesis of lymphocytes from normal cattle may have resulted from alloantigen activity in the antigen; however, normal lymph node antigen did not stimulate lymphocytes from normal adult cows or cows with lymphoma. Antigen derived from tumor was localized to the cell membrane and cytoplasm of tumor cells by indirect immunofluorescent staining using an antiserum prepared in rabbits. Specific fluorescence was reduced by the absorption of this antiserum with a crude tumor extract. This antiserum did not cross-react with normal lymph node antigen or viral components as demonstrated by immunodiffusion. With further refinement of the antigen, the blastogenesis assay may be of use in the early detection and study of the pathogenesis of lymphoma in adult cattle.

INTRODUCTION

BLV[3] is believed to be the etiological agent in the adult form of bovine lymphoma (2, 15, 17) and is oncogenic in sheep (20). The calf, thymic, and skin forms occur sporadically in younger animals and are not associated with BLV. BLV-infected cattle and sheep have complement-dependent cytotoxic antibodies to BLV-infected cells. These antibodies are of the IgG_1 subclass and are directed against the gp60 antigen which appears on the cell membrane of infected cells only in culture. They are not cytotoxic to non-virus-producing tumor cells (23). The bovine system (23) may parallel the feline leukemia system (6) since tumor cells that do not express viral antigens, and thus are not sensitive to the strong antiviral responses of the host, will be immunologically selected and give rise to tumors. It is hypothesized that those cells transformed by BLV will express a common TAA as do those cells transformed by other oncogenic viruses (8).

There are few reports of a common antigen in bovine lymphoma. Gillette et al. (7) demonstrated, by direct immunofluorescence, an abnormal antigen in tumor tissue from 11 cattle with lymphoma, using antisera prepared in calves inoculated with tumor cell suspensions. Substrates for immunofluorescence were acetone-fixed frozen sections, and specific reactivity was localized to the cytoplasm of tumor cells. Substantial cross-reactivity was present between antisera and cases of lymphoma, while normal lymphoid tissue showed no specific fluorescence. Hollinshead and Valli (11) reported a TAA that caused delayed cutaneous hypersensitivity reactions in 6 animals with lymphoma, while 3 control animals failed to respond. Reactions to normal lymph node extract were recorded in animals with lymphoma. Onuma et al. (21, 22) reported a TAA in bovine and ovine lymphoma and found antigen on tumor cell membranes and in the cytoplasm using direct immunofluorescence on acetone-fixed smears and cell suspensions of tumor cells, respectively. Antiserum used in their study was also prepared in calves inoculated with tumor cell suspensions. Animals with the calf and thymic forms had few tumor cells in which antigen was present, and several different bovine and sheep fetal cells also failed to react. The antigen was detected in clinically normal BLV-infected cattle.

The present study was undertaken to compare, by physicochemical methods, extracts of normal lymphoid tissue with tissue extracts from cases of bovine lymphoma. Immunofluorescence and immunodiffusion tests were used to localize antigen within tumor cells and demonstrate its unique nature. The tumor extract was used in a lymphocyte blastogenesis assay as a measure of cell-mediated immunity.

MATERIALS AND METHODS

Purification of Cell Membrane Proteins. Crude extracts were prepared from tissues by methods described in detail elsewhere (3, 9, 10). To summarize, tissues were minced and pushed through a wire mesh, and the cells were exposed to hypotonic NaCl solutions and then sonicated. Supernatants were collected at each step and then pooled, resulting in a soluble pool preparation. The concentration was adjusted to 10 mg protein per ml using bovine albumin standards (16). Tumor tissues of 17 adult cows with lymphoma and lymphoid tissues from 6 normal cows were subjected to this procedure. Equal volumes of the soluble pool were combined for tumor and normal animals. These were termed TSP and NSP, respectively. A special method of gradient PAG electrophoresis was used (10) to purify the individual animal extracts, TSP and NSP. Two hundred μg of sample were applied to each gel column and electrophoresed at 4 ma/column until the tracking dye was within 1 cm of the base of the gel. The gels were stained with Coomassie Brilliant Blue and scanned at 590 nm.

[3] The abbreviations used are: BLV, bovine leukemia virus; TAA, tumor-associated antigen; TSP, tumor soluble pool; NSP, normal soluble pool; PAG, polyacrylamide gel; PBS, phosphate-buffered saline [0.15 M NaCl, 0.0023 M NaH_2PO_4·H_2O, 0.007 M Na_2HPO_4·$7H_2O$ (pH 7.3)]; RPMI 1640, Roswell Park Memorial Institute Tissue Culture Medium 1640; SI, stimulation index; PHA-M, phytohemagglutinin M; AGID, agar gel immunodiffusion.

Relative migration values (R_m) were calculated for each protein band. The region of the TSP gels that contained distinct protein bands as compared with NSP gels was cut from unstained gels. The identical region from NSP gels was also collected. Proteins were eluted from these gel regions and concentrated to 500 μg protein per ml using an ultrafiltration membrane with an exclusion limit of 10,000 daltons (Amicon Corp., Lexington, Mass.). The resulting solutions were termed PAG eluate-tumor and PAG eluate-normal.

Rabbit Antiserum to PAG Eluate-Tumor. Three rabbits each received an initial dose of 500 μg of PAG eluate-tumor mixed 1:1 with Freund's complete adjuvant and given in divided doses at several intradermal sites. Two weeks later, the same dose was given in Freund's incomplete adjuvant and 50 μg were given intradermally at monthly intervals. Serum was collected 8 months after the first injection and every month thereafter. All serum samples from the 3 rabbits were pooled, filtered through a 0.22-μm membrane, and stored at $-70°$. Before use, the antiserum was heat inactivated at 56° for 30 min and absorbed with glutaraldehyde-insolubilized normal bovine serum, then equal proportions of acetone-dried normal bovine liver, spleen, and lymph node, and finally bovine buffy coat cells. The first 2 absorptions were for 18 hr at 4° and the latter for 3 hr at 4°. Those substances used for absorption were mixed in a ratio of 1:1 by volume with the antiserum. These normal tissues were derived from animals that did not possess antibody to the BLV as determined by the BLV-AGID test (Leukassay B; Pitman Moore Co., Washington Crossing, N. J.). This antiserum preparation was labeled rabbit anti-tumor antibody and was used in the immunodiffusion test. Antiserum for immunofluorescence, to localize PAG eluate-tumor, was ammonium sulphate-precipitated γ-globulin of the rabbit anti-tumor antibody. The precipitated globulin was adjusted to a concentration of 10 mg protein per ml and labeled rabbit anti-tumor γ-globulin. This antiserum detected PAG eluate-tumor in a radioimmunoassay procedure (13).

Immunodiffusion Test. The immunodiffusion test was used to examine possible cross-reactivity of rabbit anti-tumor antibody with PAG eluate-tumor, BLV antigens, and NSP. A whole virus (BLV) preparation was made from the supernatant of a fetal lamb kidney culture infected with BLV (24). Supernates of the culture, PAG eluate-tumor, and NSP were prepared at 10 mg protein per ml in PBS. The gp60 antigen used in the BLV-AGID test was rehydrated according to manufacturer's directions and was used as an antigen.

A template immunodiffusion method was done using 1% Noble agar prepared with barbital buffer (im = 0.1; 0.075 M; pH 8.6; 0.1% NaN$_3$) diluted 1:3 with distilled water. The agar film was approximately 0.6 mm thick. Slides with templates were refrigerated in a humidified chamber for 1 hr, and the wells were then filled with 40 μl of antiserum and antigens. Diffusion was allowed to proceed for 48 hr at 25° before the template was removed, and the slides were washed in several changes of PBS for 24 hr. Staining was done with 0.2% Coomassie Brilliant Blue in methanol:acetic acid solution (4.5:1) for 10 min, and slides were destained in several changes of methanol: acetic acid solution, dried in an oven at 37°, and photographed.

Immunofluorescence. The rabbit anti-tumor γ-globulin was used in an indirect immunofluorescence assay to detect antigen in cells derived from 5 cases of bovine lymphoma and 2 normal cows. Single-cell suspensions were made from tumor tissue and normal lymph node by mincing the tissue and then forcing the tissue blocks through a 250-μm mesh, using generous amounts of RPMI 1640 with 10% fetal calf serum (Lot C182118; Grand Island Biological Co., Grand Island, N. Y.). The resulting suspension was filtered through 2 layers of gauze. The cells were washed twice with PBS containing 0.02% NaN$_3$ and adjusted to 1 × 10^6 cells/ml. Air-dried smears were fixed in cold acetone for 10 min. Cell suspensions and acetone-fixed cells were incubated with the appropriate sera for 30 min at 25° and then washed 3 times in preparation for staining with a fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Lot 5504; Miles-Yeda Ltd., Elkhart, Ind.). The conjugate had been absorbed with normal bovine buffy coat cells in a 1:1 ratio of antiserum to packed cells for 3 hr at 4°. Cell suspensions and fixed cells stained with the conjugate alone served as controls. To demonstrate blocking of specific immunofluorescence, an aliquot of rabbit anti-tumor γ-globulin was absorbed with glutaraldehyde-insolubilized TSP for 3 hr at 4° and a 1:1 ratio of globulin to insoluble matrix. Stained preparations were washed in PBS 3 times. Slides were viewed using a Leitz Orthoplan photomicroscope equipped with a Tiyoda Superwide darkfield condenser (Technical Instruments Co., San Francisco, Calif.), a KP490 primary filter, a K510 barrier filter, and a HBO-200 light source. Photographs were made on Kodak Ektachrome film (400 ASA).

Lymphocyte Blastogenesis. The details of the collection and isolation of mononuclear cells and culturing techniques have been described elsewhere (12). To summarize, 2 × 10^5 cells in 200 μl culture media (RPMI 1640 supplemented with 15% fetal calf serum) were cultured in flat-bottomed wells of microtiter plates. Tests were done in triplicate and results were expressed as SI. In a pilot study, the lymphocytes from 2 cows with lymphoma and 2 normal cows were tested with varying concentrations of PAG eluate-tumor. Cells were cultured for 96 hr, 1 μCi (5 Ci/mmol) of tritiated thymidine (Amersham Corp., Oakville, Ontario, Canada) was added in 50 μl of RPMI 1640, and cultures were harvested 18 hr later. Lymphocyte cultures were repeated using the optimal dose of PAG eluate-tumor and were harvested such that the total culture period ranged from 2 to 7 days. PAG eluate-normal was used at the optimal concentration determined for PAG eluate-tumor, and lymphocytes from these 4 cows were harvested after 4 days in culture and an 18-hr labeling period. The lymphocytes from adult cows with lymphoma ($n = 13$) and clinically normal adult cows ($n = 122$) from 3 multiple case herds were tested. In addition, one animal with the calf form and 2 with the thymic form of lymphoma were tested. The lymphocytes of all animals were tested with PHA-M (Difco Laboratories, Detroit, Mich.) at the rate of 20 μg/2 × 10^5 cells. Cells were cultured for 72 hr, labeled with tritiated thymidine, and harvested 18 hr later.

Statistical Analysis. The data were analyzed using Student's t test for differences between means of triplicate PAG eluate-tumor-stimulated cultures and control cultures. The Wilcoxon Rank Sum Test with the large-sample approximation (1) was used for assessing differences in SI and differences in cpm of control or stimulated cultures.

RESULTS

Purification of Cell Membrane Proteins. The spectrophotometric tracings of TSP and NSP (Chart 1) demonstrated that

A. TUMOR SOLUBLE POOL

ABSORBANCE (590 nm)

RELATIVE MIGRATION DISTANCE

B. NORMAL SOLUBLE POOL

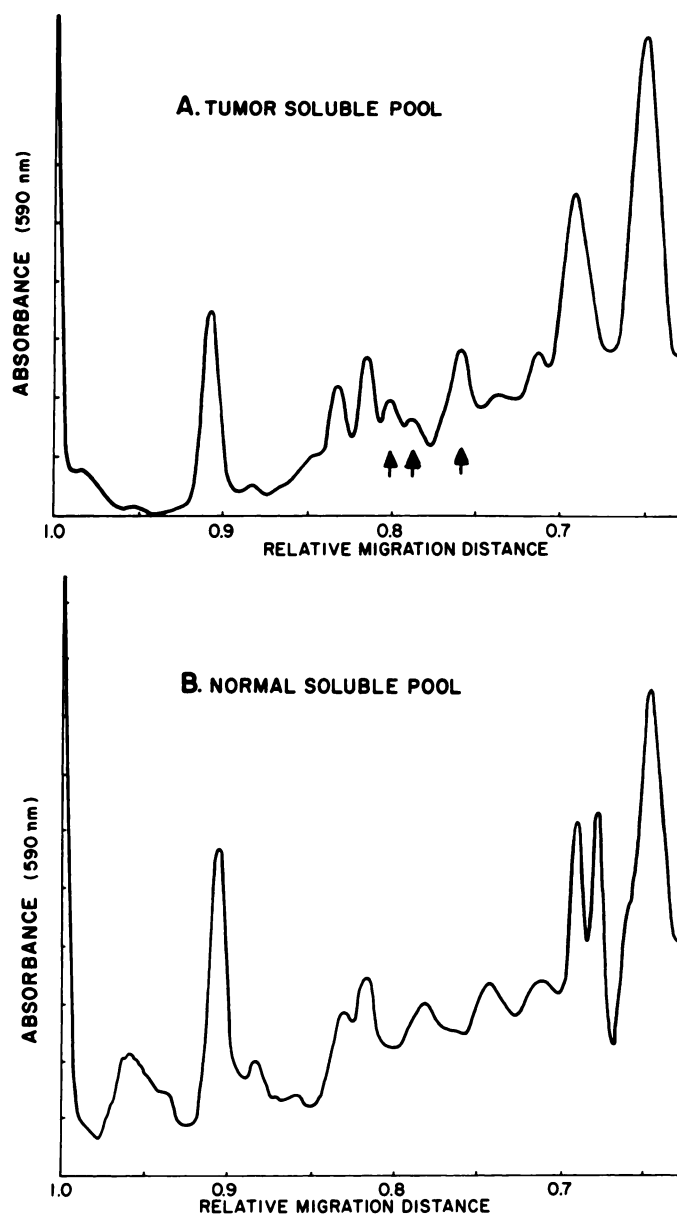ABSORBANCE (590 nm)

RELATIVE MIGRATION DISTANCE

Chart 1. Spectrophotometric tracings of PAG-separated soluble pool derived from pooled preparations of 17 cows with lymphoma (A) and 6 normal cows (B). Arrows, distinct protein bands in the TSP.

Fig. 1. Template immunodiffusion. *Center well*, absorbed rabbit anti-tumor antibody (undiluted); *Wells 1* and *4*, PAG eluate-tumor (10 mg/ml); *Wells 2* and *5*, NSP (10 mg/ml); *Well 3*, BLV (10 mg/ml); *Well 6*, BLV glycoprotein antigen (Leukassay B; Pitman Moore Co.)

the 10% gel region, from R_m value 0.67 to 0.86, possessed 3 distinct protein bands. These 3 bands were present in 8 of the 17 cows with lymphoma. Five cows with lymphoma had 2 bands while 1 band was present in 4. This gel region, as delineated by R_m values, was the source of the PAG eluate-tumor and PAG eluate-normal. There were no apparent differences in the location of protein bands below the R_m value of 0.67.

Immunodiffusion. Lines of precipitation were present between wells containing the PAG eluate-tumor and the rabbit anti-tumor antibody (Fig. 1). The antibody failed to produce precipitates with NSP or with the 2 virus preparations. On dilution of this antibody, precipitation was observable at 1:4 with PAG eluate-tumor.

Immunofluorescence. Strong fluorescence was localized to the cytoplasm of acetone-fixed tumor cells in 5 cases; however,
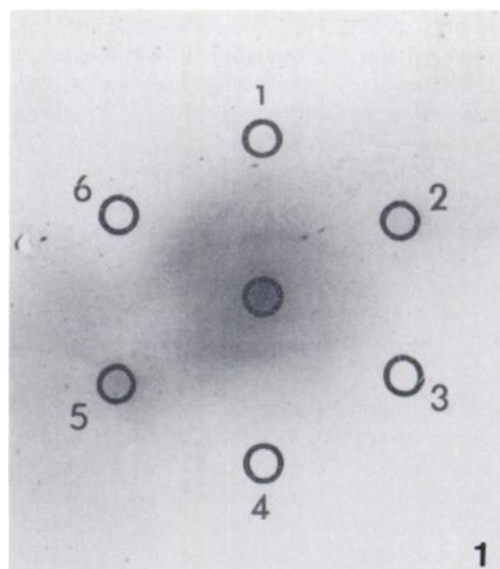
there were large numbers of disrupted cells in these preparations, and positively staining cytoplasmic debris precluded quantitative estimates. The same preparations stained with conjugate alone resulted in weak fluorescence. Acetone-fixed imprints of normal lymph node stained with rabbit anti-tumor γ-globulin and the conjugate alone resulted in approximately 20% of the cells showing weak fluorescence. Cell suspensions from 2 cows with lymphoma showed bright membrane fluorescence in approximately 60% of cells in one case and virtually all cells stained in the other (Fig. 2). When the conjugate was used alone, the percentage of positive cells was reduced to under 20% and the fluorescence was weak. The absorption of rabbit anti-tumor γ-globulin with TSP resulted in a marked decrease in the amount of bright fluorescence on tumor cells but did not eliminate it entirely.

Lymphocyte Blastogenesis. The optimal concentration of PAG eluate-tumor with 4 days in culture and an 18-hr labeling period was 10 μg/2 × 10^5 cells (Table 1). Using this concentration of PAG eluate-tumor, the culture period was varied, and it was found that 4 to 5 days in culture gave adequate stimulation (Table 2). The 4-day culture with 18-hr labeling period was adhered to in the remainder of the study. A SI to PAG eluate-tumor of 2.4 or greater was statistically significant ($p \leq 0.05$). When PAG eluate-normal was used at the same concentration and culture conditions as the PAG eluate-tumor, no stimulation was found. The SI's of the 2 cows with lymphoma were 1.4 and 0.6 and normal cows 0.6 and 1.1.

Of the 13 cows with lymphoma, the lymphocytes from 9 (69%) responded to the PAG eluate-tumor, while among clinically normal cows 5 of 122 (4%) responded (Chart 2). The difference between the group means was highly significant ($p \leq 0.0001$). The cow with the highest SI to PAG eluate-tumor was the only one in the group of cows with lymphoma detected early in the course of its disease. Responsiveness to PHA-M was decreased in cows with lymphoma ($p \leq 0.034$). Two of the 3 cows with lymphoma that did not have significant SI's to
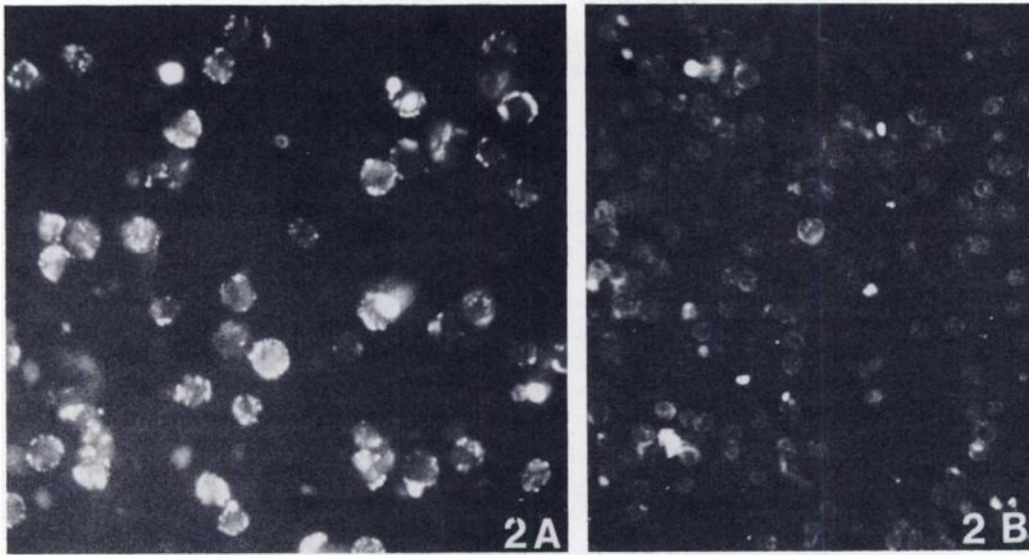
Fig. 2. Indirect immunofluorescence. *A*, tumor cell suspension stained with absorbed rabbit anti-tumor antibody and fluorescein isothiocyanate-conjugated goat anti-rabbit antiserum. × 240. *B*, tumor cell suspension stained with conjugate alone. × 150.

Table 1

Effect of concentration of PAG eluate-tumor on lymphocyte stimulation

	SI[a]		
	PAG eluate-tumor concentration (μg/2 × 10^6 cells)		
	5	10	15
Cows with lymphoma (*n* = 2)	5.0	6.5	2.8
	3.5	4.0	2.5
Normal cows (*n* = 2)	1.5	1.8	1.4
	1.8	1.6	1.6

[a] $p \leq 0.05$ if SI $\geq$ 2.4.

Table 2

Effect of culture period on lymphocyte stimulation by PAG eluate-tumor

	SI[a]					
	Days in culture[b]					
	2	3	4	5	6	7
Cows with lymphoma (*n* = 2)	0.8	1.8	3.4	2.2	1.5	1.7
	1.9	1.6	1.5	5.2	4.4	1.0
Normal cows (*n* = 2)	1.1	0.8	1.0	0.3	0.6	1.0
	0.6	1.0	0.4	0.6	0.7	0.6

[a] $p \leq 0.05$ if SI $\geq$ 2.4.
[b] Includes 18-hr labeling period.

Chart 2. Effect of PAG eluate-tumor on the stimulation of lymphocytes from cows with lymphoma and normal cows. *Vertical bars*, mean ± S.D. All animals with SI $\geq$ 2.4 (– – – –) were significantly different from control cultures ($p \leq$ 0.05).

PAG eluate-tumor had high cpm in control cultures; one was unresponsive to PHA-M and one responded normally. The 2 with elevated spontaneous uptake had lymphocytosis while 3 others with lymphocytosis had normal control cultures. The calf with lymphoma was unresponsive to PHA-M (SI = 4.2); however, the 2 animals with the thymic form of lymphoma responded to PHA-M with SI's of 25.2 and 40.5. The lymphocytes of these 3 animals were unresponsive to PAG eluate-tumor.

Prevalence of BLV Infection. All cows with lymphoma were positive on the BLV-AGID test while 45 of 122 (37%) of clinically normal individuals from multiple case herds were positive.
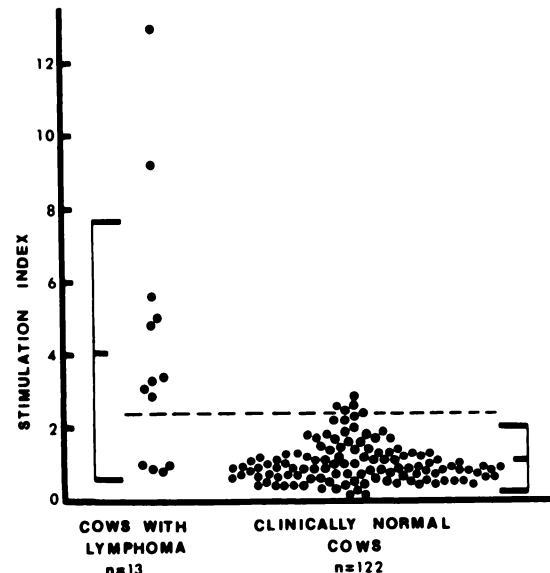
DISCUSSION

Gradient PAG electrophoresis of TSP and NSP demonstrated the differences in their protein profiles. Additional bands, made obvious by their distinct migration values, are new or altered cell membrane proteins which may result from or may be associated with neoplastic transformation. It has been established that the viral genome is not expressed in tumor cells derived from cases of bovine lymphoma (5); thus, these proteins are not viral structural proteins. The immunodiffusion studies, utilizing a highly absorbed rabbit antiserum, support the conclusion that these novel proteins are not of viral origin, and they do not occur on normal lymphocytes. These conclusions are further supported by the immunofluorescence data.

Although some fluorescence did occur in control preparations, this may be attributable to reactivity of the conjugate with surface immunoglobulin and Fc receptors. The fluorescence with tumor cells was much brighter than any of the control preparations. The cytoplasmic and cell membrane localization of the antigen is similar to other reports (21). Absorption of the antiserum with TSP substantially reduced the amount of bright fluorescence, thus supporting the immunological specificity of the reaction. Large proportions of tumor cells showed bright membrane fluorescence in 2 cases examined. Shedding of this antigen may result in the presentation of a relatively large amount to the immune system of the host, causing it to be overwhelmed or compromised; however, an attempt to demonstrate membrane antigen in the sera from cows with lymphoma was unsuccessful (13).

Cattle with persistent lymphocytosis are reported to have elevated spontaneous uptake of tritiated thymidine (19). Although 66% of cattle with lymphoma have histories of persistent lymphocytosis, only 2 of 13 cows in the present study had elevated spontaneous uptake of tritiated thymidine which correlated with lymphocytosis. Three others with lymphocytosis had normal control cultures. Thus, in the present study, lymphocytosis was not always associated with spontaneous DNA synthesis. This result may be due to this group of lymphoma cows being in the terminal stages of disease, at which time the subset of lymphocytes responsible for the spontaneous incorporation is decreased (14).

The immunological specificity of the lymphocyte blastogenesis assay was supported by the failure of PAG eluate-normal to cause the blastogenesis of lymphocytes derived from normal cows or cows with lymphoma. The lymphocytes from 9 of 13 (69%) cows with lymphoma responded to PAG eluate-tumor while only 5 of 122 (4%) clinically normal adult cows responded. The lymphocytes from 3 of 4 cows with lymphoma that did not respond were also unresponsive to PHA-M and thus were probably incapable of an *in vitro* response. The reactions in the normal cows may be attributable to alloantigen reactivity in such preparations (4). The purification of the crude extract may have reduced the rate of false positives, due to alloantigen reactivity, from a much higher figure than the 4% in the present study. Alternately, these 5 cows may be sensitized to the antigen because they have occult tumors or have spontaneously recovered from a tumor and are now immune. However, only 1 in 5 cows in this group had BLV infection, as inferred from the BLV-AGID test. Since cattle with BLV infection remain infected for life and virtually all cattle with lymphoma have BLV titers (18), then one cow in this group may not be immunologically naive to tumor. The other 4 cows may not have had titers high enough to cause precipitation in the BLV-AGID test or may have become recently infected. In the latter case, transformation must occur early in the infection in these individuals since 45 of 122 (37%) of clinically normal adult cows had antibody to the BLV and only one reacted to PAG eluate-tumor.

The results of the present study support the occurrence of a common antigen(s) among cases of bovine lymphoma and suggest that cell-mediated immunity is present and directed against this common antigen. With further refinement, this assay will aid in the determination of the rate of conversion from BLV infection to tumor development. Of particular importance will be the identification of individuals that develop tumors to characterize the early pathogenesis of the disease.

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