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Canine lymphoma-associated antigens defined by murine monoclonal antibodies

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Summary. Lymphoma in dogs resembles human non-Hodgkin's lymphoma in pathological presentation, immunophenotype, and response to therapy, thus representing a good model for comparative studies with human disease. Monoclonal antibodies (MAbs) were derived from mice immunized with a dog lymphoma cell line. Three MAbs were selected for further application in immunophenotyping and immunotherapy. The binding specificities, antigen characterization, and isotypes for these MAbs are described.

Introduction

Lymphoma is the common hemopoietic tumor in the dog. It is an autochthonous, spontaneously occurring neoplasm in an outbred animal. Most dogs present generalized lymphadenopathy and hepatosplenomegaly. Other sites of involvement include anterior mediastinal, pulmonary, intestinal, cutaneous lymph nodes and other extranodal forms [8]. The histological classification is that of the poor prognosis types in man [5]. Using the National Cancer Institute Working Formulation for Human Lymphoma Pathologic Classification [34], the majority of canine cases could be defined as high grade types. In addition, canine lymphoma responds to the same chemotherapy drugs as those used in humans i.e., prednisone, cyclophosphamide, vincristine, doxorubicin HCl, and L-asparaginase [15, 26].

Monoclonal antibodies (MAbs) against normal lymphocytes and lympoid tumors in humans have been produced by murine hybridomas [10, 22, 29, 33, 36] and have been applied clinically. Murine MAbs to cell surface antigens on canine lymphocytes have also been reported [7, 21, 23, 27, 37]. Immunophenotyping of canine lymphoma cells has shown that the majority are of B cell origin and the minority of T cell or non-T, non-B cell type [2]. This is a similar distribution to that of human non-Hodgkin's lymphomas, and canine lymphoma is a relevant model for this tumor.

We describe here the generation of MAbs with restricted binding specifity for canine lymphomas, and the characterization of the antigenic target(s).

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Materials and methods

Cell lines. Canine lymphoma cell line 17–71 was established from a lymph node of a dog with multicentric lymphoma after elutriation [13] and was maintained in minimal essential medium (MEM)/10% fetal bovine serum (FBS). Human cell lines Raji, Daudi, Molt-4, HL-60, HB5-2, K562-5, Jurkat, and U937, were maintained in L-15/15% FBS medium. All myeloma cell lines were grown in MEM/10% FBS supplemented with 8-azaguanine (15 μg/ml).

Production of hybridomas. Female BALB/c mice were immunized i.p. with 10^7 17–71 cells and 2 weeks later injected i.v. with 10^6 cells. Then 4 days after the second injection, the mice were sacrificed and their spleens separated aseptically. A spleen cell suspension was prepared as described elsewhere [19]. Immune splenocytes were fused with mouse myeloma cell lines P3X63Ag8.653 [17] or SP2/0 – Ag14 [30], as previously described [19]. Fused cells were suspended in hypoxanthine/aminopterin/thymidine medium and seeded in 24-well tissue culture plates using a feeder layer. Approximately 3 weeks after fusion, single colonies were picked from each well and tested for immunoglobulin production. Secreting hybridomas were cloned and their products tested for binding to different target cells.

Preparation of cell suspensions. Lymph nodes were surgically excised from dogs with histologically confirmed lymphoma and placed in MEM. Lymph nodes were minced and passed through a sieve (E-C Cellector) using a syringe plunger and collected into MEM. Cells were washed once with cold phosphate-buffered saline (PBS) and used immediately for FACS analysis (see below). White blood cells were purified from heparinized dog or human blood by centrifugation through Ficoll-Paque [4]. Dog fibroblasts were obtained by culture of lymph node or bone marrow cell suspension in MEM/10% FBS and adherence techniques. Cells were treated with trypsin-EDTA for 2–3 min and washed with PBS.

Direct binding radioimmunoassay. The radioimmunoassays (RIA) were performed as described elsewhere [20]. Target cells (5×10^5) were incubated with the hybridoma tissue culture supernatant containing the antibody for 1 h at room temperature. Cells were washed three times and then incubated with ^{125}I-labeled rabbit anti-mouse $F(ab^1)_2$. After 1 h, cells were washed three times and the radioactivity

present in the pellet counted in a Packard gamma spectrometer.

Determination of immunoglobulin isotype. Isotype determinations were made using a two-side amplified enzyme-linked immunosorbent assay [9, 25].

Cytofluorimetry (FACS). Live cells (5×10^5 per well) were plated in a U-bottom 96-well plate and incubated for 1 h with 50 µl of MAb (supernatant) at 4 °C on a plate shaker. Cells were washed twice with 0.1% gelatin solution in PBS without Ca^{2+} and Mg^{2+}, and 25 µl of goat anti-mouse fluorescein isothiocyanate (FITC)-conjugated IgG (Cappel Laboratories, Cochranville, Pa., USA) as added. The FITC-conjugated serum had previously been adsorbed with a cell suspension of a normal dog lymph node. After a 1 h incubation at 4° C, cells were again washed three times and resuspended in 300 µl of washing buffer. The cell suspension was then analyzed using a Cytofluorograf System 30/50 (Ortho Diagnostic Systems, Inc., Westwood, Mass., USA). Cells could be stored for 2 days after fixing with 1% paraformaldehyde for 30 min, prior to the final washes.

Immunoperoxidase assay. Tissue samples from normal and tumor-bearing dogs were cut into small pieces and frozen at −70° C or fixed in 10% neutral buffered formalin and paraffin embedded by routine procedures. Slides were deparaffinized, hydrated, and washed for 5 min in running water. Frozen sections were air dried, fixed in cold acetone for 10 min, and washed with water. Endogenous peroxidase was inhibited by treatment with 0.3% H_2O_2 in absolute methanol for 15 min [3], followed by 10% normal horse serum in PBS for 10 min. The immunoperoxidase (IP) assay was performed by a modification of the method of Hsu and Raine [14], on 5 µm sections with a biotin-avidin kit (Vector Laboratories, Inc., Burlingame, Calif., USA). The supernatant of P3X63Ag8 [18] or PBS/bovine serum albumin (BSA) buffer was used as a control.

Radiolabeling and cell extraction. Lymphoma 17–71 cells were labeled with ^{125}I by the lactoperoxidase-glucose oxidase method [28] and extracted at 4 °C for 30 min with solubilizing buffer (0.5% Nonidet P40, 140 m*M* NaCl, 10 m*M* NaF, 10 m*M* Tris, 5 m*M* EDTA, 100 kallikrein IU/ml aprotinin, 1 m*M* PMSF, pH 7.5). The extract was clarified by centrifugation at 105000 *g* for 1 h. Unlabeled cells were similarly extracted and used in immunoblotting.

Immunoblotting. After electrophoresis, proteins were transferred to nitrocellulose sheets [35] in a Trans-Blot chamber (Bio-Rad Laboratories, Richmond, Calif., USA). The nitrocellulose blots were soaked in 2% BSA in PBS and 0.1% NaN_3 overnight. The sheets were then rinsed with 2% gamma globulin-free horse serum in PBS and 0.1% NaN_3 (buffer A) and covered with hybridoma supernatant containing MAb for 1 h at room temperature. Sheets were washed three times with buffer A and incubated with ^{125}I-labeled rabbit anti-mouse Fab (approximately 2×10^5 cpm/ml) for 1 h. Nitrocellulose sheets were finally washed four times with buffer A, dried, and exposed to XAR-5 X-ray film (Eastman, Rochester, N.Y., USA) using an intensifying screen.

Immunoprecipitation. Aliquots of cleared lysates were incubated with 200 µl of hybridoma supernatant at 4 °C overnight. Immune complexes were precipitated by absorption to 100 µl of anti-mouse IgG-agarose bead suspension (SIGMA Chemical Co., St. Louis, Mo., USA). The precipitate was mixed with 60 µl of Laemmli [24] reducing buffer and boiled for 5 min. The antigens were analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis.

Electrophoresis. Electrophoresis was performed by the method of Laemmli [24] using 10% polyacrylamide with 2% SDS. Gels were stained with 0.02% Coomassie brillant blue R250 in 25% methanol-10% acetic acid, destained with 5% acetic acid and 2% glycerol, dried, and autoradiographed, or transferred to nitrocellulose sheets.

Glycolipid extraction. The total glycolipid fraction from 17–71 cells was prepared by chloroform/methanol extraction followed by separation on a SEP-PAK C_{18} cartridge (Millipore-Waters Ass. Milford, Mass., USA). Then 5 ml of chloroform/methanol/water (60:35:8 by vol.) was added to the cell pellet and the mixture sonicated at room temperature for several minutes. After centrifugation, the supernatant was evaporated to dryness using N_2. The fraction was redissolved in methanol/water (1:1 v/v) and applied to the SEP-PAK cartridge previously equilibrated in the same solvent. The cartidge was washed with 10 ml water and total glycolipids eluted with chloroform/methanol (2:1 v/v).

Thin-layer chromatography. Thin-layer chromatograms were developed on high-performance thin layer chromatography aluminum sheets (10 × 20 cm) with Silica Gel 60 (Merck, Darmstadt, FRG) using chloroform/methanol/water (60:35:8 by vol.). Anisaldehyde reagent [16] was used for detection of total glycolipids.

Chromotogram binding assay. These assays were performed as described elsewhere [11]. After chromathography, dried chromatograms were immersed in a 0.5% polyisobutylmethacrylate (Plexigum P28, Rohm GmbH, Darmstadt, FRG) solution in ether and air-dried for 5 min. Plates were sprayed and then covered with 2% BSA in PBS and 0.1% NaN_3 for 2 h. After removing the albumin solution by tipping the plates, hybridoma supernatants containing MAbs diluted 1/2 were added. The plates were incubated for 2 h in a humidified Petri dish. The antibody solutions were removed and the plates washed five times with PBS and incubated with ^{125}I-labeled $F(ab')_2$ rabbit anti-mouse Fab (approximately 1×10^6 cpm/ml) for 1 h. Finally, plates were washed six times with PBS, dried and exposed to XAR-5 X-ray film (Eastman-Kodak) using an intensifying screen.

Results

Selection of MAbs

More than 600 hybridoma colonies were established from 4 consecutive fusions; 6 hybridomas were selected, which in preliminary analysis by RIA had restricted binding specificity (Table 1). Of the 6 hybridomas, 5 secreted MAbs that bound to 17–71 cells and did not bind to human Burkitt's lymphoma Raji cells. Antibody 216-1 bound to 17–71, Raji cells and to canine fibroblasts. MAb 234 showed some crossreactivity with bone marrow-derived fibroblasts (Table 1).

Table 1. Binding of anti-canine lymphoma monoclonal antibodies (MAbs) in radioimmunoassay (RIA)

MAb		Established cell lines		
Code	Isotype	Canine lymphoma 17–71	Human Raji	Bone marrow fibroblasts (dog)
231	IgG2a	5000[a]	0	110
234	IgG1	5660	0	530
254	IgG3	5450	0	0
212	IgG1	8470	0	130
215	IgM	6650	0	0
216-1	IgM	4675	2400	2260

[a] Represents cpm of triplicate determinations minus P_3 background (usually 150–250 cpm)

As shown in Table 2, only MAb 216-1 crossreacted with human and rat cells. Very low level binding to human lymphoblastoid cells was detected for MAbs 215 (6/8), 234 (5/8), and 231 (4/8). The U937 myelomonocytic cell line, which expressed Fc receptors crossreactive with murine IgG2a immunoglobulins, also bound IgG2a MAb 231.

Binding of MAbs to normal cells

The results of FACS analysis of normal canine and human cells are shown in Table 3. Only MAb 212 crossreacted with normal canine lymphocytes; MAbs 215 and 234 showed some binding to lymphocytes and the remaining MAbs did not bind to normal canine or human lymphocytes isolated from blood. Four MAbs were clearly positive for binding to canine monocytes and three showed some binding to granulocytes. None of the MAbs bound to normal human white blood cells, except 231 which bound to 68.5% of granulocytes. The antibodies were generally negative for cells isolated from canine bone marrow and from spleen. Antibodies 212 and 215 showed high level binding with lymph node cells, while MAb 234 bound to 48.5% of lymph node-derived lymphocytes (Table 3).

Spleen, liver, and kidney samples were stained using the IP technique. MAbs 212, 215, and 216-1 showed some crossreactivity with all samples. MAb 254 bound only minimally to liver and kidney, and MAb 234 did not bind to any tissue. Antibody 231 (IgG2a) bound to hepatic duct epithelium cytoplasm only. There was no cell membrane binding in normal tissues tested by IP. Very minimal binding to renal tubule epithelial cells was occasionally observed.

Binding of MAbs to canine lymphoma cells

The lymphoma cell line 17–71 used for immunization and 15 different lymphomatous nodes were used for analysis of their phenotypes on the basis of their reactivity with 6 MAbs. In addition, 17–71 cells and malignant lymph nodes were analyzed with murine anti-human DR (IgG2a) MAb 37-7 and with anti-glycoprotein (IgG2b) MAb 480-1-4, which reacts with all human tissues.

FACS analysis of subpopulations of lymphocytes freshly isolated from lymphomatous canine lymph nodes indicated a differential distribution of binding in different animals. All 6 MAbs bound tumor cells contained in 2 lymph nodes. Except for MAb 254, all MAbs bound to 4 lymph node samples. Of the 6 MAbs, 4 bound to tumor cells derived from 2 lymph nodes (254 and 216-1 were ne-

Table 2. Binding crossreactivities of anti-canine lymphoma MAb with established cell lines of different species in RIA

MAb	Isotype	Canine lymphoma 17–71	Rat myeloma $Y_2Ag1.2.3$	Human leukemia/lymphoma							
				Raji	Daudi	Molt-4	HL60	H5B2	K562	Jurkat	U937
231	IgG2a	3845[a]	0	0	0	0	310	200	390	0	1720
234	IgG1	5190	0	0	120	140	0	185	130	0	220
254	IgG3	2075	0	0	0	0	0	100	0	0	0
212	IgG1	5265	0	0	0	0	0	0	0	0	270
215	IgM	3060	130	0	200	390	175	625	0	390	200
216-1	IgM	4545	2000	2400	2380	2490	2860	3550	2430	2410	2740

[a] Represents cpm of triplicate determinations minus P_3 background

Table 3. FACS analysis of binding of anti-canine lymphoma MAbs to normal lymphocytes of different origins

MAb	Isotype	Canine blood			Canine			Human blood	
		Lymphocyte	Monocyte	Granulocyte	Lymph node	Spleen	Bone Marrow	Lymphocyte	Granulocyte
231	IgG2a	8.8[a]	51.2	39.6	17.3	0.7	17.1	5.8	68.5
234	IgG1	34.1	69.0	15.1	48.5	3.9	12.3	5.0	9.6
254	IgG3	11.5	21.3	5.0	6.7	1.1	20.4	4.6	4.3
212	IgG1	94.1	89.9	24.2	94.6	1.3	17.7	4.4	2.8
215	IgM	34.8	60.4	8.0	93.7	2.1	17.2	5.1	3.6
216-1	IgM	5.8	12.3	7.5	8.2	1.4	NT	6.8	5.4
P3X63Ag8	IgG1	1.8	14.0	6.9	5.2	1.9	15.4	4.5	4.7

NT: Not Tested

[a] Percentage of fluorescent cells

Table 4. FACS analysis of MAbs binding to cells freshly isolated from canine lymphomatous nodes

MAb		17–71 Cells	Canine lymphoma nodes													
Code	Isotype		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
231	IgG2a	65.0[a]	93.0	22.3	3.6	3.2	33.2	74.2	53.8	23.6	30.0	29.7	16.5	19.9	62.9	5.3	84.6
234	IgGl	88.4	72.1	76.1	2.0	54.4	11.3	27.7	54.8	22.7	22.3	6.5	11.7	33.9	8.0	0.8	71.8
254	IgG3	26.5	1.8	21.0	2.1	28.6	2.2	8.4	22.3	31.3	8.1	8.0	13.3	10.4	8.4	0.6	8.1
212	IgGl	96.8	99.8	9.4	80.7	82.3	94.1	98.2	88.5	96.3	86.8	68.9	76.9	28.8	9.2	94.9	95.9
215	IgM	96.8	99.8	11.4	76.8	84.5	86.2	96.8	77.5	86.6	67.7	81.5	94.0	30.0	12.0	84.8	97.6
216-1	IgM	3.9	4.9	25.3	10.7	14.2	35.9	24.7	60.5	67.7	29.0	73.3	85.1	29.1	6.0	0.3	8.0
37-7	IgG2a	60.6	NT	NT	NT	NT	NT	NT	85.3	90.7	80.6	72.8	83.4	21.2	57.6	75.7	90.9
480-1-4	IgG2b	NT	NT	NT	NT	NT	NT	NT	22.2	NT	6.2	4.9	9.2	3.2	NT	15.8	16.9
P3X63Ag8	IgGl	1.1	1.0	5.2	3.0	4.5	3.0	4.0	7.0	NT	19.7	4.9	9.9	6.0	1.0	5.3	1.0

NT: Not Tested
[a] Percentage positive cells

gative, Table 4). The remaining 6 canine lymph node-derived tumor cells had binding with dissimilar patterns, 2 of which were reactive only with MAbs 212 and 215, which might be directed against DR-related molecules (Table 4).

Table 5 summarizes the reactivity of the 6 MAbs with canine lymph node tumor cells. Of the 9 lymph nodes screened with (anti-DR) MAb 37-7 all showed binding reactivity comparable to MAbs 212 and 215, suggesting that these 2 MAbs may be directed against DR molecules on canine lymphocytes.

Antigen analysis

As presented in Table 6, MAbs 212 (IgGl) and 215 (IgM) immunoprecipitated a 29×10^3 dalton protein. Antibody 234 (IgGl) immunoprecipitated a 36×10^3 dalton protein. Antibodies 231 (IgG2a), 254 (IgG3), and 216-1 (IgM) did not bind to immunoblots of tumor cell extracts and no protein molecules were immunoprecipitated from the tumor or normal cells. No binding of those MAbs to the glycolipid extracts of tumor and normal cells was detected.

Table 5. Summary of binding of anti-canine lymphoma MAbs with malignant canine lymph node cells

MAb	Ratio Positive/total	%
231	11/15	73.3
234	10/15	66.6
254	4/15	26.7
212	13/15	86.6
215	13/15	86.6
216-1	10/15	66.6

Table 6. Summary of antigens detected with anti-canine lymphoma MAbs

Antibody	Antigen detected	
	Immunoprecipitation	Glycolipid fractions
231	None	Negative
234	36×10^3 daltons	Negative
254	None	Negative
212	29×10^3 daltons	Negative
215	29×10^3 daltons	Negative
216-1	None	Negative

Discussion

Canine lymphoma resembles human non-Hodgkin's lymphoma in pathological presentation, response of tumor cells to the same cytotoxic agents, correlation of immunophenotyping of cell surface markers to histological classification and response to therapy, and in distribution of B, T, and non-T, non-B cell lymphomas [2, 6]. It therefore represents a good model for comparative studies with human disease. For these purposes, we have obtained hybridomas secreting MAb from mice immunized with an established canine lymphoma cell line (17–71).

At least three of these MAbs have unique binding specificities to canine lymphoma. MAb 231 (IgG2a) bound to 73% of lymphomas tested. This antibody is cytotoxic in antibody-dependent cell cytotoxicity tests (data not shown) and seems to be a promising candidate for immunotherapy [12, 32]. The minimal crossreactivity of MAb 231 observed with the human U937 myelomonocytic cell line and with monocytes and granulocytes is probably due to the binding of IgG2a protein to the Fc receptors expressed by these cells [1]. MAb 234 (IgG1) also has restricted specificity, binding to about 70% of canine lymphomas and could be used to select an isotype switch variant from IgG1 to IgG2a [31] for immunotherapy. The third antibody, MAb 254 (IgG3) bound only to about 27% of lymphomas tested, but it has very restricted binding specificity. This MAb is also a potential candidate for isotype switch variant selection, since it may find therapeutic application in a subpopulation of lymphomas negative for MAbs 231 and 234. All three antibodies show promise as diagnostic classification tools of canine lymphomas.

MAbs 212 and 215 appear to detect the canine Ia-like or DR antigen with a molecular weight of 29×10^3 daltons Deege, et al. [7] described a similar murine MAb that recognized an Ia-like antigen of mononuclear cells consisting of a bimolecular complex with 29 and 34×10^3 dalton components. Eight of nine dogs screened with MAb SK37-7, a murine anti-human DR antibody, and with MAbs 212 and 215 showed similar binding patterns, suggesting that the common target of these MAbs is an Ia-like molecule.

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