Identification of Monoclonal Antibodies for Immunohistochemical Staining of Feline B Lymphocytes in Frozen and Formalin-Fixed Paraffin-Embedded Tissues

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ABSTRACT

Commercially-available monoclonal antibodies to B lymphocytes were evaluated for immunohistochemical staining of feline B lymphocytes in frozen and formalin-fixed, paraffin-embedded tissues using an avidin biotin complex immunoperoxidase immunohistochemical technique. Three monoclonal antibodies: F46A and F72A raised to "carnivore" B lymphocytes and RA3.6B2 raised to murine B lymphocytes, stained B lymphocyte-dependent areas of frozen feline lymphoid tissue. In addition, antibody RA3.6B2 stained B lymphocyte dependent areas in formalin-fixed, paraffin-embedded feline tissues. There was no staining of T lymphocyte-dependent areas in either frozen or formalin-fixed tissues. Dual parameter flow cytometry, using an anti-pan-T lymphocyte antibody, revealed that greater than 99% of the cells stained by RA3.6B2 are a population distinct from T lymphocytes. F46A was shown to stain a sub-population of those cells stained with RA3.6B2. These antibodies may be useful in the identification of feline B lymphocytes using immunohistochemistry and flow cytometry and thereby provide additional tools to study B lymphocyte ontogeny and the significance of lymphocyte phenotype in lymphoid neoplasia in cats.

RÉSUMÉ

Des anticorps monoclonaux (AcMo) anti-lymphocytes B disponibles commercialement ont été évalués pour étudier les lymphocytes B félins dans des sections de tissus congelés ou fixés à la formaline puis enrobés de paraffine, par une technique immunohistochimique (IHC) utilisant la révélation du complexe avidine-biotine par l'immunoperoxidase. Les régions lymphocytes B-dépendantes des tissus lymphoïdes félins congelés ont été mises en évidence par trois AcMo : F46A et F72A étant dirigés contre les lymphocytes B de carnivores et RA3.6B2 est dirigé contre les lymphocytes B murins. De plus, l'AcMo RA3.6B2 a réagi avec les lymphocytes dans la région B-dépendante de tissus félins fixés à la formaline et enrobés de paraffine. Aucune cellule dans la région T-dépendante des tissus congelés ou fixés à la formaline n'a réagi avec ces anticorps. L'utilisation d'un anticorps anti-pan-lymphocytes T lors d'analyse par cytométrie en flux (CF) a démontré que plus de 99% des cellules réagissant avec RA3.6B2 appartiennent à une population cellulaire distincte des lymphocytes T. L'AcMo F46A a réagi avec une sous-population de cellules mises en évidence par RA3.6B2. Ces anticorps pourraient s'avérer utiles pour identifier les lymphocytes B félins par IHC et CF et ainsi fournir des outils additionnels pour l'étude de l'ontogénèse des lymphocytes B et de l'importance du phénotype des lymphocytes impliqués dans les néoplasmes lymphoïdes chez les chats.

(Traduit par docteur Serge Messier)

INTRODUCTION

Monoclonal antibodies directed against cell surface molecules enable classification of lymphocytes into functional subsets and help define stages of differentiation and activation. Production of feline lymphocyte-specific monoclonal antibodies has lagged behind development of these antibodies for use in human medicine, but is now progressing, in part due to the potential of feline retroviral-induced immunodeficiency diseases as models of human disease (1,2,3). Recent reports describe anti-feline T lymphocyte monoclonal antibodies that recognize the feline equivalent of CD 4 and CD 8 molecules found on helper and cytotoxic T lymphocytes respectively (1,2,3,4), and two monoclonal antibodies which recognize multiple subsets of feline T lymphocytes (1,2,4).

There are few reports of monoclonal antibodies that identify feline B lymphocytes. The earliest report describes a series of monoclonal antibodies which detect cytoplasmic heavy and light chains of feline immunoglobulin (5). These antibodies identified pre-B lymphocytes as cells expressing cytoplasmic μ chain without surface immunoglobulin. A more recent report describes a monoclonal antibody that binds CD45, and identifies B lymphocytes and a subset of T lymphocytes (6), as well as a monoclonal antibody to a CD 21-like molecule which recognizes B lymphocytes and follicular dendritic cells (7). No antibodies have been described that identify feline B lymphocytes over a broad range of developmental stages.

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TABLE 1. Species of immunizing B lymphocyte, sources, and working dilutions of monoclonal antibodies tested for immunohistochemical staining of feline lymph nodes

Monoclonal antibody	Species of immunizing B lymphocyte	Dilutions used	Source
LN-1 (22)	human	undiluted; 1:10	ICN[a]
LN-2 (22)	human	undiluted; 1:10	ICN[a]
B-4 (23)	human	1:50, 1:200	Coulter[b]
B-1 (24)	human	1:50; 1:200	Coulter[b]
BA-1 (25)	human	1:50; 1:200	Boerhinger-Mannheim[c]
HD-6	human	1:10; 1:50	Boerhinger-Mannheim[c]
HD-37	human	1:10; 1:50	Boerhinger-Mannheim[c]
4KB5	human	1:25; 1:50	Dako[d]
To15	human	1:25; 1:50	Dako[d]
L26	human	1:25; 1:100	Dako[d]
F72A (19)	carnivore	1:10; 1:50	VMRD[e]
F46A (19)	carnivore	1:10; 1:50	VMRD[e]
RA3.6B2 (15)	mouse	1:200; 1:500	Cedarlane[f]

[a] ICN Biomedical, Costa Mesa, CA
[b] Coulter Corp, Hialeah, FL
[c] Boerhinger — Mannheim, Montreal, PQ
[d] Dako Corp, Carpinteria, CA
[e] Veterinary Medical Research and Development, Pullman, VA
[f] Cedarlane Laboratories Ltd., Mississauga, ON

Anti-feline lymphocyte antibodies have been studied using either flow cytometry or immunohistochemistry on frozen tissue sections; none have been shown to be effective for immunohistochemical staining of formalin-fixed, paraffin-embedded tissues. Antibodies reactive in fixed-tissue immunohistochemistry are valuable in retrospective studies and studies in which morphologic detail is important.

Since conservation of epitopes present on the surfaces of lymphocytes has been demonstrated among species (8,9), the goal of this study was the evaluation of 13 commercially-available anti-B lymphocyte monoclonal antibodies, which identify B lymphocytes of various species, for immunohistochemical labeling of feline B lymphocytes in frozen and formalin-fixed, paraffin-embedded tissue.

MATERIALS AND METHODS

Tissues were obtained from three healthy domestic short hair cats, that tested serologically negative for feline leukemia virus (FeLV) antigen and feline immunodeficiency virus (FIV) antibodies (CITE COMBO, Idexx corp. Portland, Maine), following euthanasia with intravenous barbiturate (in accordance with the Canadian Council for Animal Care "Guidelines for the Care and Use of Experimental Animals"). Cats 1 and 2 were between 3 and 6 mo of age and cat 3 was mature. Spleen, mesenteric lymph node, thymus, liver and skin were collected from all cats; brain, thyroid, salivary gland, stomach, jejunum, colon, heart, lung, adrenal gland, pancreas, kidney and skeletal muscle were also collected from cat 3. Samples of tissue were cut into 1 cm^3 blocks and frozen in OCT (Tissue-Tek, Miles, Elkhart, Indiana) at −70°C for cryostat processing or fixed in 10% neutral buffered formalin for variable periods of time: 24 h, 48 h, 72 h, 1 wk or 1 mo, then embedded in paraffin wax.

Antibodies (Table 1) were initially evaluated for immunohistochemical staining of B lymphocyte-dependent areas of 6 micron cryostat (Cryocut E, Reichert-Jung) sections of lymph node tissue, on poly-D-lysine (MWt 239,300 Sigma, St. Louis, Missouri) coated slides, from cats 1 and 2 using a robotic slide stainer (Code-On Immuno/DNA slide stainer, Fisher Scientific, Edmonton, Alberta) and an avidin-biotin immunoperoxidase staining technique previously described (10). Controls included serial sections of lymph nodes similarly stained, but with omission of the primary antibody and substitution with isotype matched irrelevant monoclonal antibodies. Cytospin preparations of a human chronic lymphocytic leukemia cell line SA-4 (donated by Dr. Skinnider, Cancer Research Centre, Saskatoon, Saskatchewan) were used as positive controls. Cytospins were prepared (Cytospin 2 Shandon, Pittsburgh, Pennsylvania) at 500 rpm for 10 min on poly-D-lysine (Sigma, St. Louis, Missouri) coated slides, fixed in acetone for 15 s and stored at −70°C until processed for immunocytochemistry. Antibodies that stained the B lymphocyte-dependent areas (11) in frozen sections were further tested on the panel of frozen tissues and on the panel of formalin-fixed, paraffin-embedded tissues. Formalin-fixed tissues were tested with and without pretreatment of the sections with a proteolytic enzyme, a 15 min exposure to 0.01% protease type XIV (Sigma Chemical Co., St. Louis, Missouri) at 37°C and the enzyme retrieval system TUFS (Monosan, Uden, The Netherlands) used according to the manufacturer's directions.

Frozen slides were stained according to the following protocol: the tissue sections were fixed in 99.5% acetone (BDH, Toronto, Ontario) for 5 min and then briefly rinsed in reagent alcohol (95% ethanol, BDH, Toronto, Ontario). The slides were then moved through four steps where they were alternately immersed in automation buffer (10X Autobuffer, Biomeda, Foster City, California; 100 mL acetone, 5 mL 15% v/v BRIJ 35 [23 Lauryl ether, Fisher Scientific, Edmonton, Alberta] final pH 7.5, 1L distilled water) for approximately 30 s and then blotted dry. Normal serum, 4% (from the species in which the biotinylated antibody was raised) diluted with Dulbecco's PBS (1M, pH 7.2, Gibco, Grand Island, New York) was then applied for approximately 30 s and the slides incubated for 12 min at 42°C . The primary anti-B lymphocyte antibody was applied to the slides for approximately 30 s and the slides incubated at 42°C for 1 h. Following this, the slides were rinsed 3 times with automation buffer and blotted dry between each rinse step. The appropriate secondary antibody was applied (1:400 horse anti-mouse IgG, or 1:100 rabbit anti-mouse IgM, or 1:100 rabbit anti-rat IgG/IgM, [Dimension Laboratories, Mississauga, Ontario]) followed by a 30 min incubation at 42°C. The slides were then rinsed in the same manner as following application of the primary antibody. Slides were next placed in a solution containing: 50 mL Dulbecco's

PBS, 2 mL 50% v/v H_2O_2, 5 mL 10% w/v Na azide, and 50 μL 15% BRIJ 35 for approximately 30 s, blotted dry then placed in the same solution for 15 min. The slides were blotted dry and rinsed with automation buffer for 4 rinses. The avidin biotin complex (Vectastain Elite ABC, Dimension Laboratories, Mississauga, Ontario) was applied for 30 s followed by a 30 min incubation at 42°C. The slides were again rinsed and dried as prior to the application of the ABC solution. The chromagen, Diaminobenzidene-4HCl (DAB, Electron Microscopy Science, Fort Washington, Pennsylvania) 1 mg/mL in 5 mL Dulbecco's PBS supplemented with 10 μL H_2O_2, was applied for 30 s and the slides incubated for 5 min at 42°C. The slides were rinsed in a solution containing: 1 L distilled water, 150 mL methanol and 5 mL 15% BRIJ for 12 s then blotted dry. This rinse/blot step was repeated twice. The slides were then counterstained using Gill's hematoxylin III (EM Diagnostic Systems, Gibbstown, New York) with 100 μL 15% BRIJ 35 for 12 s. The slides were blotted and the stain reapplied. The slides were then rinsed/blotted twice with the same solution used prior to the counterstain. Finally, the slides were rinsed with automation buffer, blotted dry and rinsed with the methanol/BRIJ solution.

The formalin-fixed, paraffin-embedded tissues were also stained using an automated technique as follows: the slides were first treated with Hemo D (Fisher Scientific, Edmonton, Alberta) as follows: a 3 min application of Hemo D followed by a 10 min incubation in a 65°C oven and a drying step; then an 18 s application and 3 min incubation; 6 s application followed by drying, and 2 more 6 s applications with a drying step in between each application. The slides were then rinsed twice in reagent alcohol, with drying steps in between. This was followed by an application of 1% v/v H_2O_2 in methanol and a drying step. Again the slides were rinsed twice in reagent alcohol as above. Automation buffer was then applied for 12 s and the slides blotted dry followed by a single rinse in reagent alcohol and 2 subsequent rinses in automation buffer. A blocking step followed in which the slides were placed in 4% normal serum (corresponding to the species in which the biotinylated antibody was raised) in Dulbecco's PBS for 30 s and then incubated at 42°C. The primary anti-B lymphocyte antibody was then applied and the slides incubated at 42°C for 55 min. This was followed by 3 rinses in automation buffer. The appropriate biotinylated secondary antibody (1:100 rabbit anti-rat IgG/IgM, 1:400 horse anti-mouse IgG, [Dimension Laboratories, Mississauga, Ontario]) was applied for 30 s and the slides incubated for 35 min at 42°C. The slides were then rinsed 3 times in automation buffer. The chromagen was applied next. Diaminobenzidene-4HCl 1 mg/mL in 5 mL Dulbecco's PBS with 10 μL H_2O_2 was applied for 18 s and then the slides incubated for 5 min at 42°C. Slides were rinsed 3 times in a solution containing; 1 L distilled water, 150 mL methanol, and 5 mL 15% BRIJ 35. The counterstain, Gill's Hematoxylin III with 100 μL Tween 20 (polyoxyethylenesorbitan monolaurate, Sigma, St. Louis, Missouri) and 100 μL 15% BRIJ 35 was applied next followed by 2 rinses in the water/methanol/BRIJ solution. Finally, slides were rinsed in automation buffer for 1 min followed by a final rinse in the water/methanol/BRIJ solution. Slides were evaluated, using light microscopy, for dark brown granular deposition of chromagen. The T lymphocyte-dependent areas were similarly demonstrated, in serial sections, by immunohistochemical staining with a polyclonal antisera, at dilutions of 1:400 and 1:800, to the CD3 molecule (Dako Corp, Carpinteria, California) (12).

Dual parameter flow cytometric analysis was performed using phycoerythrin labeled RA3.6B2 versus CF255 (a pan-T lymphocyte antibody, VMRD, Pullman, Washington) or F46A which were detected using fluorescein conjugated goat anti-mouse IgG (Southern Biotech, Birmingham, Alabama). The details of the methodology were as follows: 60 μL of cells from peripheral blood buffy coat, collected by jugular venipuncture in EDTA, at a concentration of 3.6×10^7/L were placed in wells in a V-bottom microtitre plate and 10 μL of normal goat serum was added. The plates were then incubated for 30 min at 4°C. Seven μL of the primary antibody, either CF255 or F46A, diluted 1:10 in 4% normal goat serum in PBS was then added to each well and the plates incubated 20 min at 4°C. The plates were then washed 3 times with 100 μL, then 200 μL twice using fluorescence activated cell sorter (FACS) wash (Dulbecco's PBS with 1% bovine serum albumin, 5 mM EDTA and 0.01% Na Azide). Plates are spun at 1000 rpm for 6 min, without braking, between washes. Cells were then resuspended in 100 μL of a 1:200 dilution of goat anti-mouse IgG FITC in PBS +Tween 20 with 4% normal goat serum and incubated for 20 min at 4°C then washed with FACS wash as outlined previously. Five μL of normal rat serum and 50 μL of normal goat serum in PBS was added to each well and the plates incubated for 10 min at 4°C. Next, 10 μL of a 1:10 dilution of RA3.6B2-PE (phycoerythrin) was added to the wells and incubated for 10 min at 4°C followed by the previously described FACS wash. Finally, 200 μL of the cell suspension was added to 500 μL of 1% paraformaldehyde in PBS and analyzed by FACS sort (Biorad, Mississauga, Ontario) with lymphocyte gates determined using forward and side scatter.

RESULTS

Three of 13 monoclonal antibodies, F46A, F72A and RA3.6B2 stained B lymphocyte-dependent areas (11) in frozen sections of feline lymph node and spleen. There was dark staining of cells in the follicle centres and moderate staining of the mantle zone with antibody F46A (Fig. 1). Staining with antibody F72A was uniform and confined to the mantle zone (Fig. 2). The majority of the cells, including most discernible lymphocytes, throughout the entire lymphoid follicle were darkly stained with antibody RA3.6B2 in both frozen and formalin-fixed tissues (Figs. 3 and 4). None of the monoclonal antibodies immunohistochemically stained T lymphocyte-dependent areas or non-lymphoid cells in the thymus or spleen (11). Neither was there evidence for specific staining of non-lymphoid cells in other tissues and organs. Antibody F72A, however, did exhibit moderate diffuse non-specific background

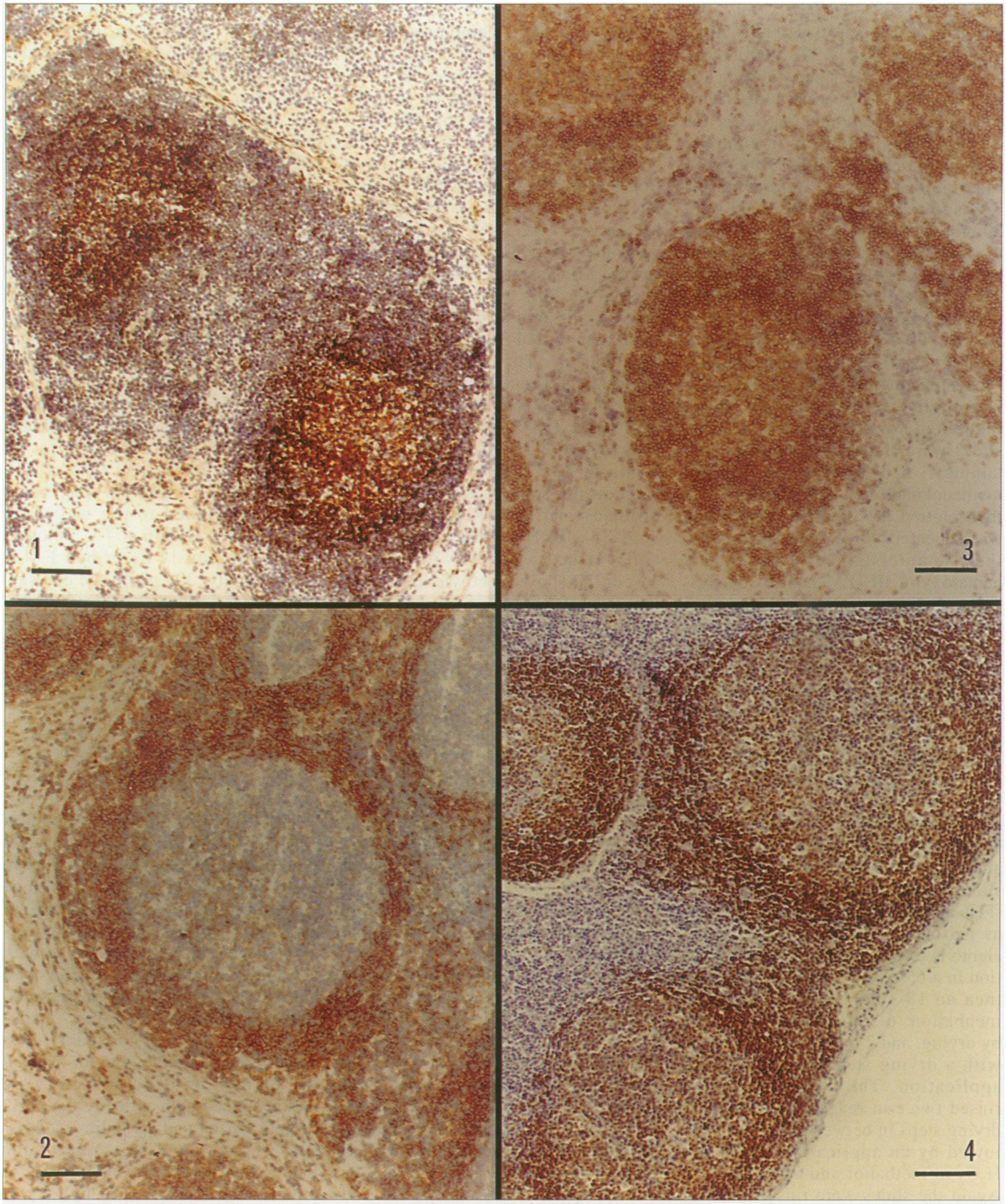

Figure 1. Section of frozen feline lymph node. There is dark staining of the lymphocytes in the germinal centres of the lymphoid follicle with moderate staining of cells in the mantle zone. Avidin biotin peroxidase complex method, with Gill's hematoxylin counterstain. The primary antibody is the monoclonal antibody F46A. Bar = 100 microns. Figure 2. Section of frozen feline lymph node. There is dark staining of the cells in the mantle zone of the lymphoid follicle with occasional dark staining of cells in the germinal centres. Avidin biotin peroxidase complex method with Gill's hematoxylin counterstain. The primary antibody is the monoclonal antibody F72A. Bar = 100 microns. Figure 3. Section of frozen feline lymph node. There is dark staining of the cells throughout the entire lymphoid follicle. Avidin biotin peroxidase complex method with Gill's hematoxylin counterstain. The primary antibody is RA3.6B2. Bar = 100 microns. Figure 4. Section of formalin-fixed paraffin-embedded feline lymph node. There is dark uniform staining of the cells throughout the lymphoid follicle. Avidin biotin peroxidase complex method with Gill's hematoxylin counterstain. The primary antibody is RA3.6B2. Bar = 100 microns.

staining in some tissues, particularly epithelial tissues.

Dual parameter flow cytometry demonstrated that greater than 99% of cells labeled by RA3.6B2 were lymphocytes not detected by CF 255, consistent with B lymphocyte recognition (Fig. 5). Monoclonal antibody F46A was shown to label a sub population of the lymphocytes labeled by RA3.6B2 (Fig. 6).

The epitope detected by RA3.6B2 was susceptible to prolonged formalin fixation with a progressive decrease in staining intensity following 24, 48 and 72 h fixation in formalin prior to paraffin-embedding. Antigen retrieval using proteolytic enzyme treatment was ineffective, however pretreatment with TUFF (Monosan, Uden, The Netherlands) enabled evaluation of tissues that had been fixed for much longer time periods, up to and including 1 mo.

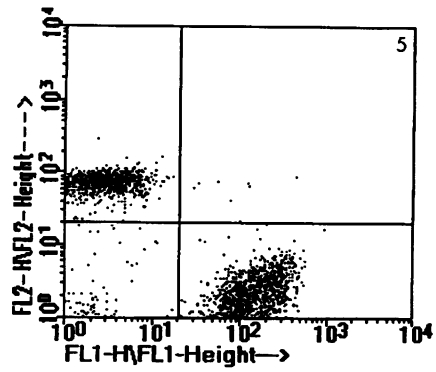

Figure 5. Dot plot of dual parameter flow cytometry using phycoerythrin labeled monoclonal antibody RA3.6B2 (FL2) and FITC labeled anti-pan-T lymphocyte monoclonal antibody CF 255 (FL1) in ficoll hypaque separated feline peripheral blood, as detected by specific log fluorescence intensity. There is mutually exclusive staining consistent with B and T lymphocytes.

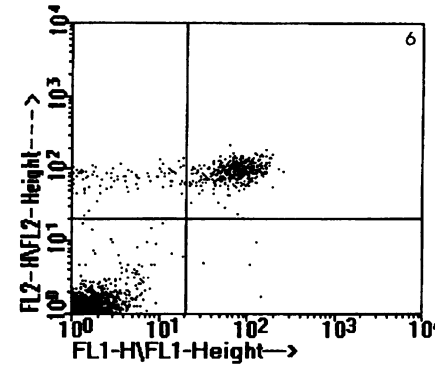

Figure 6. Dot plot of dual parameter flow cytometry using phycoerythrin labeled monoclonal antibody RA3.6B2 (FL2) and FITC labeled monoclonal antibody F46A (FL1) in ficoll hypaque separated feline peripheral blood, as detected by specific log fluorescence. There is affinity of antibody F46A for a sub population of lymphocytes labeled with RA3.6B2.

DISCUSSION

This study identifies 3 commercially-available monoclonal antibodies that react with cells in the B lymphocyte-dependent (11) areas in frozen sections of feline lymph node and spleen, one of which, antibody RA3.6B2, recognizes feline B lymphocytes in formalin-fixed tissue.

Antibody RA3.6B2 detects an epitope of the B220 antigen of the CD45 family of high molecular weight glycoproteins (6,14,15,16,17). CD45 designates a group of cell surface glycoproteins (14) identified as receptor-like protein tyrosine phosphatases which are expressed exclusively on nucleated hemopoietic cells (17). Monoclonal antibodies (like RA3.6B2) that react with a restricted subgroup of these surface glycoproteins are classified as specific for CD45R. The polymorphisms within the CD45 designation are thought to result from differential splicing of mRNA transcripts (14). The distinct outer membrane-domain structures have unique ligand specificities and are thus somehow related to lymphocyte function. The monoclonal antibody RA3.6B2 has been thought to recognize murine B cells exclusively (17), however there is recent evidence to suggest that the epitope recognized by RA3.6B2 may also be expressed in a small proportion of blasted murine T lymphocytes of splenic origin in addition to B lymphocytes (14). Results of the present study support RA3.6B2 recognition of predominantly feline B lymphocytes. A small proportion (less than 0.1%) of cells in the peripheral blood co-stain with a pan-T lymphocyte marker, CF 255, and RA3.6B2. Further studies are required to identify the epitope detected by the antibody in cats and to relate the morphologic findings to the range of functional lumphocyte subset(s) expressing this epitope.

The CD groupings of antibodies F46A and F72A are unknown. Antibody F46A appears to detect a subset of B lymphocytes (18) based on the restricted staining pattern within lymphoid follicles and dual label flow cytometry. Antibody F72A, although known to detect other leukocytes, including monocytes and granulocytes, in addition to B lymphocytes using flow cytometry (18), reveals an immunohistochemical pattern of staining in lymphoid tissue consistent with recognition of an epitope present only on a subset of B lymphocytes. It may be that epitope density or accessability results in differential detection between these two assays. The potential for the use of these antibodies to better characterize feline B lymphocyte subsets warrants further evaluation.

Immunophenotyping of lymphoid malignancies has prognostic and diagnostic significance in human disease (20). Lymphoid tumors account for 30% of all feline malignancies (21). T lymphocyte tumors carry a poorer prognosis than B lymphocyte tumors in people and in dogs (19,20). Prognosis can be related to feline leukemia virus and feline immunodeficiency virus status, but there are no consistent correlations with other factors that have been examined, such as anatomic site (21). Paucity of monoclonal antibodies for the identification of feline B lymphocytes has precluded evaluation of prognosis with respect to lymphocyte subset phenotype. Identification of a commercially-available monoclonal antibody that reacts with feline B lymphocytes in tissue is significant for basic and applied research. These anti-B lymphocyte antibodies coupled with available anti-feline T lymphocyte antibodies (1,7) will enable retrospective and prospective studies to better understand normal lymphocyte kinetics in the cat as well as alterations in the lymphocyte subsets which occur secondary to neoplasia and diseases, such as FIV and FeLV.

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