Evaluation of monoclonal antibodies for identification of subpopulations of myeloid cells in bone marrow obtained from dogs

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Objective—To evaluate monoclonal antibodies that may be useful for immunophenotyping myeloid cells in bone marrow of dogs.

Sample Population—Bone marrow specimens obtained from 5 dogs.

Design—Specimens were labeled with monoclonal antibodies that detected CD18, major histocompatibility antigen class-II (MHC class-II), CD14, and Thy-1. Cells labeled with each of the antibodies were isolated by use of a fluorescence-activated cell sorter. Differential cell counts of sorted cells were used to determine cells that were labeled by each of the various antibodies.

Results—Myeloid cells labeled with anti-CD18 antibody included granulocytes, lymphocytes, and monocytes-macrophages. Immature and mature granulocytes were labeled. Lymphocytes, monocytes-macrophages, and eosinophils were labeled with anti-Thy-1 antibody. Cells labeled with anti-MHC-class II antibody included approximately 9% of bone marrow cells, which consisted almost exclusively of lymphocytes and monocytes-macrophages. Approximately 4% of bone marrow cells were labeled with anti-CD14 antibody, with >90% of sorted cells being monocytes-macrophages.

Conclusions and Clinical Relevance—Four monoclonal antibodies for use in detecting subpopulations of canine bone marrow cells were evaluated. These antibodies should be useful in differentiating the origin of leukemic cells in dogs. (Am J Vet Res 2001;62:1229–1233)

Cytologic evaluation of bone marrow of dogs is complicated because of the large number of cell types and the various stages of development within these cell types. Monoclonal antibodies provide powerful tools to differentiate leukocytes through detection of lineage-specific cell-surface markers. Monoclonal antibodies are particularly useful in determining the origin of hematopoietic malignancies, because cellular immaturity and atypical features confuse identification of cell lineage. However, little is known about the distribution of cell-surface antigens for various developmental stages of myeloid cells in canine bone marrow.

A limited number of monoclonal antibodies that react with mature canine leukocytes have been described. Most of these antibodies react with various subtypes of canine T lymphocytes. Few antibodies have been tested on canine bone marrow cells. Anti-Thy-1, a pan-T-lymphocyte marker, reportedly labels 15% of canine bone marrow cells. The Thy-1 antigen is highly expressed on T lymphocytes, monocytes, and some B lymphocytes but weakly expressed on granulocytes. Anti-major histocompatibility (MHC) class-II antibody labels a subpopulation of canine bone marrow cells. When canine leukocytes were analyzed, T and B lymphocytes and monocytes were labeled. The anti-CD34 antibodies reportedly label 1 to 3% of canine bone marrow cells. These cells are characterized as radiation-resistant colony-forming cells and, therefore, probably represent hematopoietic stem cells.

The purpose of the study reported here was to evaluate the subtypes of myeloid cells that were labeled with a battery of monoclonal antibodies. To identify labeled cells, suspensions of bone marrow cells were incubated with each monoclonal antibody, and fluorescent cells were isolated by use of fluorescence-activated cell sorting.

Materials and Methods

Collection and processing of bone marrow specimens—Bone marrow specimens were obtained from 5 consecutive clinical canine bone marrow samples of dogs that were submitted to the Cytology Service at the University of Minnesota Veterinary Teaching Hospital. Clinical diagnoses for these dogs included immune-mediated thrombocytopenia, granulocytic chlirchosis, immune-mediated hemolytic anemia, pure RBC aplasia, and cutaneous mast cell tumor. Bone marrow samples had been aspirated into syringes rinsed with 2% EDTA solution. Wedge-type smears were prepared and stained with a modified Wright stain. Differential cell counts were performed on 500 cells for each of 2 smears, and mean values were calculated. Total nucleated cell counts were determined for each bone marrow specimen by use of a hemacytometer.

After counts were performed, 3 x 10^7 nucleated bone marrow cells were placed in each of six 6-ml sterile plastic tubes, and erythrocytes were lysed by addition of 2 ml of erythrocyte lysis buffer. Mixtures were incubated for 10 minutes at 24°C, tubes were centrifuged, and the cell pellet was washed and resuspended in 100 µl of Dulbecco PBS solution containing 1% sheep serum and 2 mM sodium azide. Cell suspensions were incubated with 200 µl of specific monoclonal antibody or with isotype-matched irrelevant monoclonal antibody (anti-human thyroglobulin) for 60 minutes. Each antibody was adjusted to a protein concentration of 10 µg/ml. Antibodies included anti-bovine CD18 (IgG1), anti-equine Thy-1 (IgM), and anti-equine MHC class-II (IgG1). After incubation on ice for 60

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minutes, cells were washed, resuspended in 100 µl of Dulbecco PBS solution containing 1% sheep serum and 2 mM sodium azide, and incubated with 100 µl of a 1:10 dilution of fluorescein-conjugated sheep anti-mouse IgG or rat anti-mouse IgM on ice for 30 minutes. Thereafter, samples were washed, diluted to a final volume of 1.0 ml, and analyzed within 3 hours after preparation.

Another aliquot of bone marrow cells was incubated with anti-human CD14 that had been conjugated with phycoerythrin-Cy5. This antibody reportedly reacts with canine monocytes but not other leukocytes. After incubation on ice for 60 minutes, samples were washed, resuspended in 1.0 ml of Dulbecco PBS solution containing 1% sheep serum and 2 mM sodium azide, and analyzed within 3 hours after preparation.

**Fluorescence-activated cell sorting**—Flow cytometric analysis was performed, using a flow cytometer. Cell populations were displayed by use of forward and side-angle light scatter properties and as scatter plots with forward-angle light scatter on the x-axis and fluorescence intensity on the y-axis. Using bone marrow specimens labeled with isotype-matched irrelevant control antisera (anti-human thyroglobulin), gates were set so that < 0.5% of cells were included in the gate. A minimum of 30,000 fluorescent cells was sorted. Sorted cells were centrifuged and resuspended in 500 µl of Dulbecco PBS solution containing 10% sheep serum. Then, 50 to 200 µl of cell suspension was centrifuged in a cytocentrifuge, and slides were stained with modified Wright stain. Differential cell counts of 500 cells were determined on each of 2 slides. For these counts, the investigator did not have knowledge of the antibody used for cell sorting.

**Cytochemical staining**—For some slides of bone marrow samples, granulocytes were differentiated from monocytes by detecting prominent peroxidase activity. Peroxidase staining was performed by use of a commercial cytochemical analysis kit.

**Results**

**Anti-CD18**—Labeling of bone marrow cells with anti-CD18 antibody resulted in distinct populations of fluorescent and nonfluorescent cells (Fig 1). Percentage of positive cells detected in each of the 5 dogs was 51.3, 71.1, 73.8, 78.0, and 80.2%. When percentage of sorted cells was compared with percentage of cells in the original sample, all myeloid cells were substantially enriched, whereas erythroid cells were severely reduced or totally lacking (Table 1). All developmental stages of granulocytes were increased, indicating that the antibody reacted with immature as well as mature granulocytes. Percentages of lymphocytes and monocytes-macrophages also were enriched.

**Anti-Thy-1**—Labeling of bone marrow cells with anti-Thy-1 antibody resulted in populations of fluorescent and nonfluorescent cells; however, some cells had intermediate fluorescence (Fig 1). Sorted cells represented 12, 13, 17, 18, and 21% of the total number of bone marrow cells analyzed for each of the 5 dogs. Sorted cells were substantially enriched for lymphocytes, monocytes-macrophages, and eosinophils (Table 2). Noneosinophil granulocytes and erythroid cells were in low numbers or totally lacking in sorted populations. We attempted to eliminate eosinophils in 2 samples of canine bone marrow by adjusting the gate to detect only highly fluorescent cells. This resulted in a decrease in the percentage of eosinophils, but the percentage of eosinophils was still > 15% of the total number of cells sorted.

**Anti-MHC class-II**—Labeling of bone marrow cells with anti-MHC class-II antibody resulted in distinct populations of fluorescent and nonfluorescent
Table 1—Expression of CD18 by bone marrow cells obtained from 5 dogs with various hematologic conditions that were evaluated by use of fluorescence-activated cell sorting

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Dog 1</th>
<th>Dog 2</th>
<th>Dog 3</th>
<th>Dog 4</th>
<th>Dog 5</th>
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<tr>
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Values represent percentage of each cell type detected before (initial) and after (sorted) cell sorting on the basis of fluorescence intensity.

Table 2—Expression of Thy-1 by bone marrow cells obtained from 5 dogs with various hematologic conditions that were evaluated by use of fluorescence-activated cell sorting

<table>
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<tr>
<th>Cell type</th>
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<tr>
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<td>3</td>
<td>37</td>
<td>1</td>
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<tr>
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See Table 1 for key.

Table 3—Expression of major histocompatibility class-II by bone marrow cells obtained from 5 dogs with various hematologic conditions that were evaluated by use of fluorescence-activated cell sorting

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<tr>
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<tr>
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See Table 1 for key.

Table 4—Expression of CD14 by bone marrow cells obtained from 5 dogs with various hematologic conditions that were evaluated by use of fluorescence-activated cell sorting

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<th>Cell type</th>
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<td>Monocytes-macrophages</td>
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</tbody>
</table>

See Table 1 for key.
Canine T and B lymphocytes appear to express MHC
sively of lymphocytes and monocytes-macrophages. 


cells (Fig 1). Sorted cells represented 3, 8, 9, 10, and 12%
of the total number of bone marrow cells for the 5 dogs. Sorted cells were highly enriched for lympho-
cytes and monocytes-macrophages (Table 3). Granulocytes and erythroid cells were severely deplet-
ed or totally lacking among sorted cells.

Anti-CD14—Labeling dog bone marrow cells with anti-CD14 antibody resulted in a small population of fluorescent cells of variable size. Sorted cells represented 1, 3, 3, 5, and 6% of the total number of bone mar-
row cells for each of the 5 dogs. Sorted cells were highly enriched for monocytes-macrophages (Table 4). Percentage of granulocytes was determined by quanti-
tation of peroxidase-positive cells. Few or no granulo-
cytes or erythroid cells were detected among the sort-
ed cells; however, lymphocytes were detected in 4 of 5 dogs. When examined by use of light microscopy, sorted cells resembled macrophages and had phagocy-
tized material in their cytoplasm. Eight to 19% of the cells resembled monocytes.

Discussion

Analysis of results of the study reported here indicated that 4 monoclonal antibodies can be used to
detect subpopulations of leukocytes in bone marrow obtained from dogs. Three of these antibodies are com-
ercially available, and specificity for binding of puta-
tive cell-surface markers has been established.1,9,14 Specificity of the anti-CD18 antibody for detection of CD18 in canine cells has not been evaluated. However, staining of all myeloid cells, but not erythroid cells, is
consistent with cellular distribution of CD18.

All myeloid cells in bone marrow, including gran-
ulocytes, lymphocytes, and monocytes-macrophages,
were labeled by anti-CD18 antibody. Immature and mature granulocytes were labeled, indicating that the marker is expressed early in granulocyte development. In other studies,1,9 investigators have documented that CD18 is a panleukocyte marker for dogs. Therefore, this antibody should be useful in differenti-
ating myeloid cells from erythroid cells. Extensive cross-species reactivity of antibodies directed toward CD18 has been documented;1 therefore, commercially available anti-human CD18 antibodies have a high probability of cross-reacting with canine CD18.

The anti-Thy-1 antibody labeled 12 to 21% of bone marrow cells. This percentage is in agreement with results of another study.2 In the study reported here, lymphocytes, monocytes-macrophages, and eosinophils were labeled by anti-Thy-1 antibody. Other investigators have indicated that Thy-1 is a pan-T-lymphocyte marker, but it is strongly expressed by monocytes and some B lymphocytes and is weakly expressed on granulo-
cytes.5,7 Labeling of canine eosinophils limits the useful-
ness of anti-Thy-1 antibodies for detection of lympho-
cytes and monocytes-macrophages in bone marrow.

The anti-MHC class-II antisera labeled 5 to 12% of canine bone marrow cells that consisted almost exclu-
sively of lymphocytes and monocytes-macrophages. Canine T and B lymphocytes appear to express MHC class-II antigens. In 1 study, 98% of canine lympho-
cytes were labeled by anti-MHC class-II antisera.10,30 Therefore, this antibody appears to be useful for detection of cell populations of lymphocytes and monocytes-macrophages in canine bone marrow.

The anti-CD14 antisera labeled 1 to 6% of canine bone marrow cells, with > 90% of sorted cells being monocytes-macrophages. Analysis of these data indicated that CD14 is highly lineage specific for mono-
cytes-macrophages. However, in 4 of 5 dogs, a few lymphocytes appeared to express CD14. It has been docu-
mented that CD14 is expressed on monocytes but not lymphocytes or granulocytes.32 Most sorted cells had morphologic features that resembled mature macrophages, with < 20% resembling monocytes. This
distribution may reflect the relative numbers of mono-
cytes and macrophages in canine bone marrow. Monocytes are rarely detected in normal bone marrow, whereas macrophages are evident in low number.30 However, promonocytes may not be detected by use of light microscopy because of their similar appearance to neutrophilic myelocytes and metamyelocytes.30

Immunophenotypic evaluation of bone marrow is
an important tool in evaluation of acute-onset leukemias, myelodysplastic syndromes, and non-
Hodgkin’s lymphomas in humans.3,9,13 However, the
technique suffers from lack of sensitivity and specifici-
ty of antibodies.1 Coexpression of surface markers on various cell lines appears to be typical and may be increased in disease conditions. For example, CD4 (a T-lymphocyte marker) is expressed by some erythroid progenitors in humans and is expressed by many canine neutrophils.34 Of further concern is expression of aberrant surface markers in myeloproliferative dis-
ese.3 To avoid these pitfalls, it has been recommend-
ed that 2 or more antibodies be used for determination of cell lineage. Therefore, to provide a reliable pheno-
type for leukemias in dogs, additional lineage-specific antibodies will be needed.

Results of the study reported here revealed that 4 monoclonal antibodies may be useful for phenotyping myeloid cells obtained from the bone marrow of dogs with various hematologic disorders. These antibodies provide a pan-myeloid marker, lymphocyte and mono-
cyte-macrophage marker, and specific monocyte-
macrophage marker. Antibodies that detect additional markers, particularly markers that differentiate mature and immature granulocytes, may be needed for immunophenotypic analysis of myeloproliferative dis-
orders in dogs.

1Diff Quik stain, Baxter Healthcare Corp, McGaw Park, Ill.
2Erythrolyse solution, Serotec USA, Washington, DC.
3DAK-Tg6, IgG1, DAKO Corp, Carpinteria, Calif.
4TuK4, Serotec USA, Washington, DC.
5MCA 1095, Serotec USA, Washington, DC.
6MCA 1085, Serotec USA, Washington, DC.
7Leukocyte peroxidase kit, Sigma Chemical Co, St Louis, Mo.
8MCA 199, Serotec USA, Washington, DC.
9R 15.7, provided by Jutila M, Department of Veterinary Molecular Biology, College of Agriculture, Montana State University, Bozeman, Mont.
10DH 2A, VMRD Inc, Pullman, Wash.
11MCA 1085, Serotec USA, Washington, DC.
12MCA 1095, Serotec USA, Washington, DC.
13MCA 199, Serotec USA, Washington, DC.
14TuK4, Serotec USA, Washington, DC.
15FACS Calibur flow cytometer, Becton-Dickinson Co, Pleasanton, Calif.
16Cytospin, Shandon Southern Products LTD, Cheshire, UK.
17Leukocyte peroxidase kit, Sigma Chemical Co, St Louis, Mo.
References