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A Novel Monoclonal Antibody Against Canine Monocytes/Macrophages

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ABSTRACT

The production and partial characterization of a monoclonal antibody, the IgG_1 IH1, which recognizes an antigen distributed in canine monocytes/macrophages, is reported here. The distribution and apparent molecular weight of the antigen recognized by the IH1 MAb was determined in peripheral blood leukocytes, peripheral blood monocyte-derived macrophages and tissue sections of spleen, liver and skin, using Western blotting, immunocytochemistry, immunohistochemistry and flow cytometry. The IH1 MAb-recognized antigen was detected in Western blotting under non-reducing conditions spread out as a large band covering the position corresponding to the migration of molecules with molecular weights from 55 to 73 kDa. The IH1 MAb labeled blood monocytes, tissue macrophages in lymph nodes, and in the mantle zone of the spleen, and Kupffer cells in the liver. It did not react with human cells. In flow cytometric analysis, the IH1 MAb reacted with a subpopulation of monocytes. The MAb described herein may become a valuable tool for diagnosis and research on canine diseases.

INTRODUCTION

Besides their importance as companion and working domestic animals, dogs have become a useful animal model in biomedical research. Dogs are used in studies on transplantation and on infections diseases such as leishmaniasis and many diseases that also occur in human beings. For instance, dogs spontaneously develop a variety of diseases such as allergic asthma,[1] penfigus, systemic lupus eritematosus, and congenital or acquired immunodeficiences,[2] providing an adequate model for research.

Monocytes/macrophages are the major target cells for most intracellular pathogens.[2] These cells also play important roles in graft rejection[3] and in the development of the immune response in zoonosis such as leishmaniasis.[4] Nevertheless, the panel of cell markers for the identification of different macrophage populations in dog is restricted.

Macrophages are widely distributed throughout body tissues, showing a high plasticity and constituting a heterogeneous cell population. Depending on their localization, macrophages show differences in their morphology, function and antigen expression. Moreover, macrophages may be in different maturation and activation stages.[5]

Monoclonal antibodies with specificity against canine leukocytes have been reported previously. However, only a limited panel of canine cell markers are available.[6–10] These antibodies react with many cell types, especially lymphocytes and/or granulocytes. However, antibodies that react with canine monocytes and macrophages are rare.[11–13]

The production and partial characterization of a monoclonal antibody binding to canine monocytes/macrophages but not to granulocytes and lymphocytes is reported here. Its successful uses in immunohistochemical and flow cytometric analysis are also reported.

MATERIALS AND METHODS

Animals and tissues

Two normal 24–36-month-old outbreed dogs, weighing 15 kg, were used throughout the study. The dogs were kept separately, under standard care and feeding conditions, in the ken-

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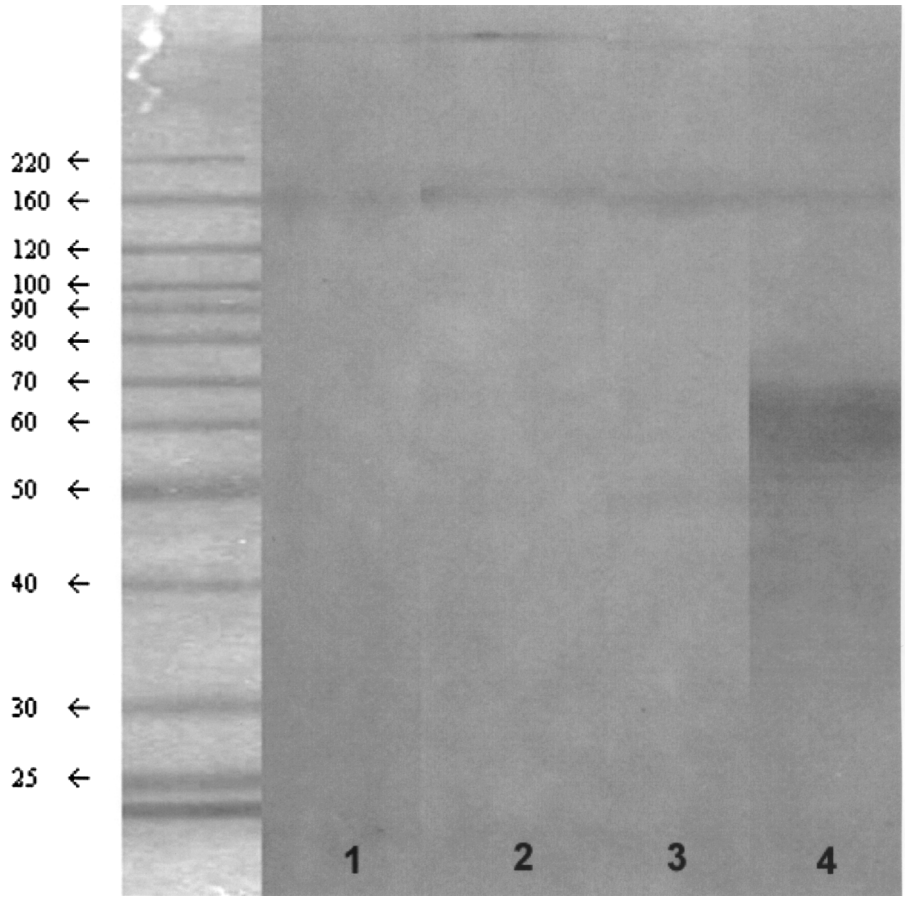

FIG. 1. Western blot of canine PBMC lysates on nitrocellulose membrane, stained with the IH1 monoclonal antibody (MAb). Nitrocellulose strips were incubated with saline (1); normal mouse serum (2); isotype matched control (3), and IH1 MAb (4). The positions of molecular weight markers, stained with Comassie blue, are shown on the left.

nel of Gonçalo Moniz Research Center, Oswaldo Cruz Foundation and used as blood donors. Normal dog tissues were obtained from the Department of Pathology and Clinics, Veterinary School, Federal University of Bahia. These specimens were derived from necropsy of animals brought to the ambulatory for various reasons. Ten six-month old, female BALB/c mice, maintained under standard conditions, from the breeding stock of the Gonçalo Moniz Research Center, were used for immunization with dog antigens. All the experiments were conducted according to the Oswaldo Cruz Foundation guidelines for animal experimentation.

Antigen preparation and monoclonal antibody production

The procedure for the monoclonal antibody production is detailed elsewhere.[14] Briefly, canine blood leukocytes were used for immunizing BALB/c mice, the splenocytes of which were fused with SPO_2 myeloma cells (Sp20-Ag14), following standard procedures.[15] Hybrid cells showing antibody activity against canine leukocytes were screened by ELISA[14] and the antibody-producing cells were subjected to three subsequent rounds of subcloning steps, by limiting dilution, in order to obtain stable hybridomas. Culture supernatants were used in subsequent experiments. The isotype of the MAbs was determinated using a mouse monoclonal antibody isotyping kit (monoclonal antibody-based mouse Ig isotyping kit; B&D PharMingen, Los Angeles, CA). The assay was performed following the protocol recommended by the manufacturer.

Immunoprecipitation analysis and Western blotting

Canine spleen cells were suspended to 5×10^6 cells/mL^{-1} in 0.15 M phosphate-buffered saline, pH 7.2 (PBS), subjected to 12 20-sec sonification bursts (Sonifier 450 Brandson, USA) and incubated in a 100 mM NaCl, 1 mM PMSF water solution, for 1 h at 4°C. The insoluble fraction was removed by centrifugation at 500*g* for 30 min. The MAb was coupled to protein A-Sepharose beads (Pharmacia, Uppsala, Sweden). One-milliliter volume of all spleen cell extracts were incubated with 100 μL of protein A-Sepharose beads for 1 h. The supernatant was then incubated with 100 μL of MAb-coated Sepharose beads for 1 h at 4°C, with continuous rotation. The beads were washed with PBS containing 0.05% of Tween 20 (PBS-T20), resuspended with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (62.5 mM Tris-HCl, 2% w/v SDS in 10% v/v glycerol/water) and removed by centrifugation. The protein A–Sepharose immunoprecipitated

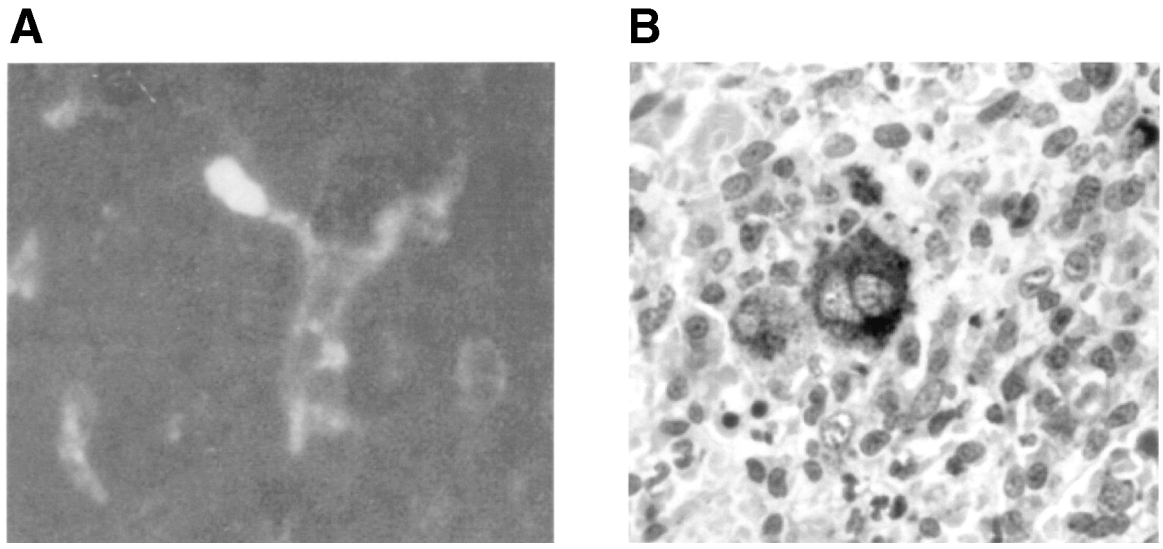

FIG. 2. Immunofluorescence of canine liver and immunoperoxidase staining of canine spleen by the IH1 monoclonal antibody (MAb). In liver, the MAb stained Kupffer cells (**A**), and in the spleen the IH1 MAb stained megakaryocytes (**B**). Original magnification, ×400.

antigens were run in SDS-PAGE under reducing (with 2-mercaptoethanol) and non-reducing (without 2-mercaptoethanol) conditions, as described by Harlow & Lane.[15] Proteins were then electrophoretically transferred to nitrocellulose membrane and incubated with the hybridoma supernatants, diluted 1:2, in PBS-T20 containing 5% skimmed milk powder (w/v; PBS-T20-milk), followed by successive incubations with peroxidase-conjugated sheep anti-mouse immunoglobulin polyclonal antibodies (Sigma Chemical Co., St. Louis, MO) and a mixture of 3,3′-diaminobenzidine (DAB, Sigma Chemical Co.) and H_2O_2 in PBS. A normal mouse serum dilution containing approximately 25 μg of immunoglobulin per mL of PBS-T20-milk was used as negative control.

Indirect immunofluorescence

Immunofluorescence reactions were carried out on Tween 20-permeabilized or non-permeabilized canine and human PBMC. Cryostat sections (6–8 μm in thickness) of normal canine lymph node, spleen, liver, skin, and kidney were also tested by indirect immunofluorescence. PBMC or tissue sections were air-dried and fixed in alcohol for 5 min at room temperature (RT). After three washes in PBS, the tissue sections and PBMC were incubated for 30 min at 37°C with hybridoma supernatants diluted 1:2 in PBS or PBS-T20-milk. The detergent was used for membrane permeabilization to guarantee the access of the MAb to cytoplasm epitopes. Irrelevant antibody, diluent and normal mouse serum (diluted 1:500) were used as negative controls. The slides were then washed three times in PBS and incubated for 30 min with fluorescein isothiocyanate (FITC)–conjugated sheep IgG anti-mouse immunoglobulins (Sigma Chemical Co.) diluted 1:200 in 1% Evans Blue in PBS. After washing three times with PBS for 10 min, the slides were mounted with glycerol buffer and examined by UV microscopy.

Immunohistochemical staining

Samples from spleen and lymph nodes were collected from a healthy mongrel dog that had been sacrificed with an overdose of phenobarbital. The same cryostat sections of canine tissues used in immunofluorescence were also tested in immunohistochemical assay. A normal lymph node was also collected from a mouse killed by excess anesthesia. The tissues were placed in neutral buffered 10% formalin for 12 h, and embedded in paraffin wax. Serial 5–8-μm-thick sections were cut, placed on poly-L-lysine (Sigma Chemical Co.)–coated slides, and allowed to dry at room temperature. The slides were dewaxed in xylene, rehydrated through graded alcohols to distilled water and subjected to a heat-mediated antigen retrieval treatment, following the protocol described by Shi et al.[16] Briefly, the slides were placed into a plastic bag filled with 0.01 M sodium citrate buffer, pH 6.0, and heated in a steamer for 30 min. Once cooled, the heat-treated slides were washed twice for 5 min each in PBS. Before staining, endogenous peroxidase was inhibited by incubation with H_2O_2 and sodium azide in PBS.[16] Non-specific binding was blocked by pre-incubation of the slides with 10% normal rabbit serum and bovine serum albumin in PBS. The hybridoma supernatants were then applied onto the slides and left overnight at 4°C. After two washes of 5 min with PBS, the slides were incubated with a 1:600 dilution of a biotin-rabbit anti-mouse immunoglobulin conjugate (DAKO, Capinteria, CA) for 45 min at 37°C. The sections were then incubated for 30 min with a 1:500 dilution of a streptoavidin–peroxidase conjugate (Pierce, Chemical Co., Rockford, IL). After two washes with PBS, the peroxidase activity was developed with a mixture of 25 $\mu g \cdot mL^{-1}$ of 3,3-diamine-benzidine (Sigma Chemical Co.) and 0.05% H_2O_2 in PBS for 2 min. The slides were then washed in distilled water, counterstained with hematoxylin, gradually dehydrated with alcohol and mounted in Canadian balsam (Riedel de Haen AG, Hannover, Germany). Either, the control section lacked primary antibody, or it was replaced by an irrelevant isotype-matched MAb.

In vitro macrophage differentiation

Canine PBMC were adjusted to the concentration of 5×10^6/mL in RPMI medium supplemented with 20% heat-inacti-

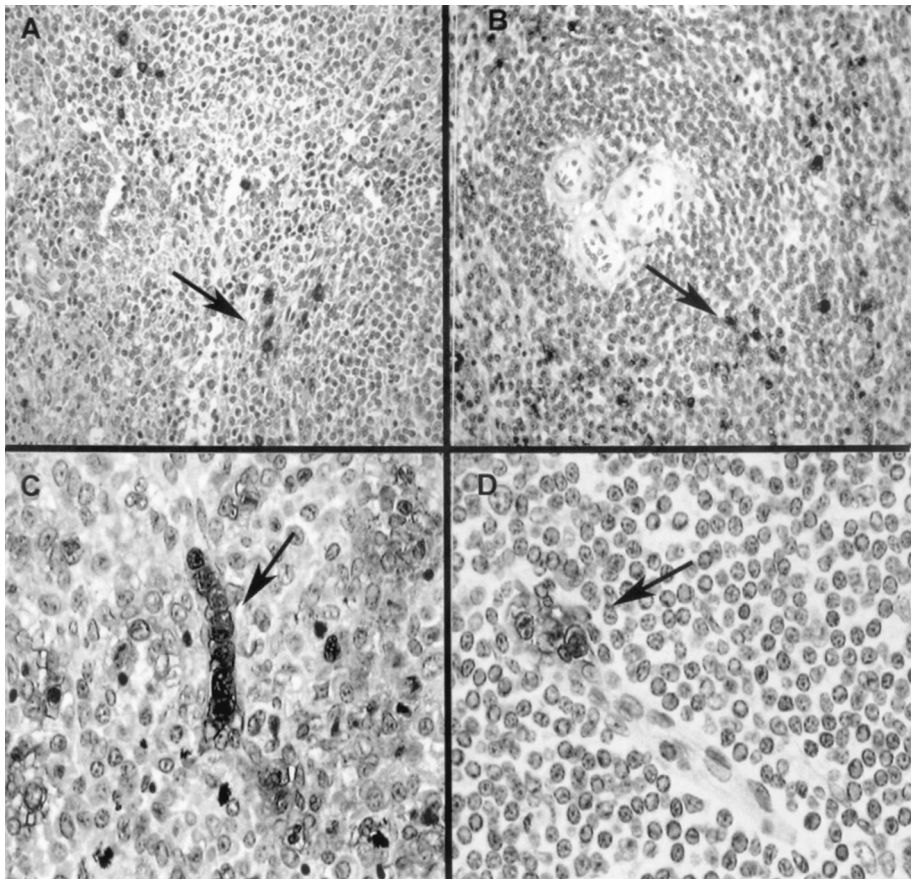

FIG. 3. Immunoperoxidase staining of spleen (**A,C**) and lymph node (**B,D**) by the IH1 MAb in formalin-fixed, paraffin-embedded sections. In the spleen, the IH1 MAb stained macrophages in the mantle zone and some scattered macrophages in the red pulp, as well as megakaryocytes. It did not mark macrophages in the lymphoid areas. In lymph nodes, the IH1 MAb labeled macrophages in the marginal sinus, some dispersed macrophages in the paracortical area, and in the medullar cords. The arrows indicate macrophages (A,B), and monocytes (C,D). Sections were counterstained with hematoxylin. Original magnification, ×200 (A,B), ×400 (C,D).

vated fetal bovine serum, and a cell suspension was delivered into flat-bottomed chamber slide or culture dishes (Nunc, Naperville, IL), for 24 h, and carefully washed. The adsorbed cells were further incubated for five to ten days. The monocyte-derived macrophages adhered to the chamber slide bottoms, were used in indirect immunofluorescence tests using the MAb and control immunoglobulin as described above. To perform cytometric analysis, the adherent cells were detached from the Petri dish with cell scrapers, washed three times with washing solution and the expression of surface antigen were analyzed by cell cytometry, as described below.[3]

Flow cytometry

Ten milliliters of blood, collected by venipuncture of the cephalic vein, was incubated at room temperature, for 15 min, with 40 mL of a hemolytic solution (8.26 g of NH_4Cl, 1 g of $KHCO_3$, and 0.037 g of Na_4EDTA per liter of distilled water). The suspension was centrifuged at 700*g* for 1 min and the supernatant removed. The cells were washed three times with washing solution (0.1% NaN_3 and 5% BSA in PBS) and adjusted to the concentration of 2×10^7/mL. 100 μL of the cell suspension was delivered into wells of 96-well microplate (Nunc, Naperville, IL), incubated with a 1:2 dilution of the hybridoma supernatant for 20 min at 4°C, and washed three times. FITC-conjugated sheep anti-mouse immunoglobulin (Sigma Chemical Co.), diluted 1:200 in PBS, was added to the sediment and incubated for 20 min at 4°C. The plate was centrifuged and cells were resuspended in 500 μL of washing solution prior to analysis in a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Negative control samples were stained with the secondary antibody only. This procedure, including the buffers for indirect immunofluorescence labeling, was recommended by the Canine Leukocyte Antigen Workshop (CLAW).[17]

For two-color analysis, the cells were incubated with MAbs against canine CD4 (rat anti-canine CD4 monoclonal antibody, Serotec, England), and CD8 (rat anti-canine CD8 monoclonal antibody, Serotec, Oxford, England). Free binding sites were blocked with 5% heat-inactivated normal canine serum.

Biotinylation of the MAbs was carried out using NHS-biotin (Vector Laboratories, Burlingame, CA) according to the protocol suggested by the supplier. The reactions with the IH1, anti-CD4, and anti-CD8 MAbs were detected using FITC-conjugated sheep anti-rat immunoglobulin. Reactivity with the biotinylated MAbs was detected using B-phycoerythrin-conjugated streptavidin (Sigma Chemical Co.).

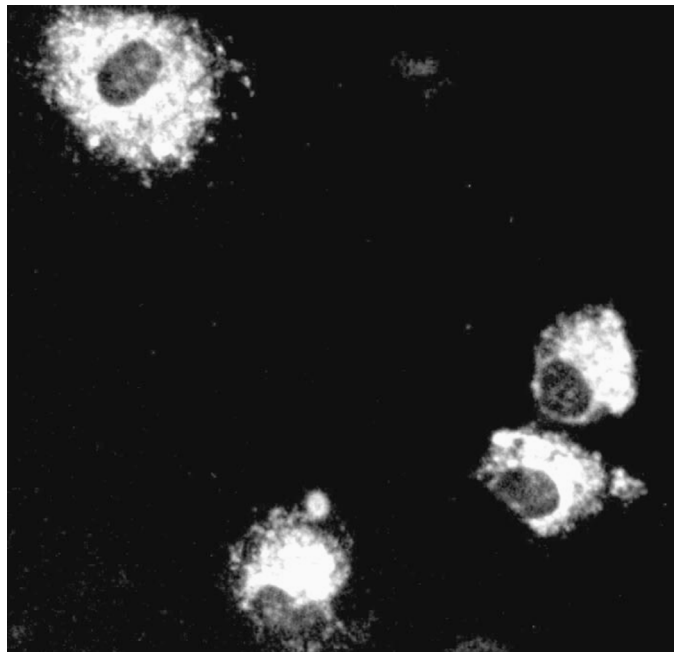

FIG. 4. Canine peripheral blood monocyte-derived macrophages, mature macrophages in cell cultures stained by indirect immunofluorescence. Large adherent cells with elongated projections stained by the IH1 MAb. Morphological appearance on day 8. Original magnification, ×400.

RESULTS

Molecular weight of the antigen recognized by IH1 MAb

Under non-reducing conditions, the IH1 MAb recognized a broad protein band spread out between the position corresponding to molecules with molecular weights of 55 and 73 kDa, in Western blotting using canine leukocyte PBMC (Fig. 1). Under reducing conditions, no positive bands were identified by the IH1.

Antigen distribution on peripheral blood and lymphoid tissue cells by immunofluorescence and immunohistochemistry

The tissue distribution of the antigens recognized by the IH1 MAb was analyzed by immunofluorescence and immunohistochemical staining on different canine tissues.

Monocytes were the majority of cells stained by IH1 MAb when a single cell suspension of canine PBMC was tested by immunofluorescence. The staining pattern was diffuse and homogeneous, in the presence or absence of membrane permeabilization with Tween-20.

The staining pattern of the IH1 MAb on canine tissue sections was similar with immunohistochemistry and immunofluorescence. The MAb also stained cells on formalin-fixed, paraffin embedded tissue sections.

The IH1 MAb identified a cell population of the mononuclear phagocytic system in the spleen and lymph nodes. In the spleen it stained macrophages in the mantle zone and some scattered macrophages in the red pulp, as well as megakaryocytes (Fig. 2). It did not stain macrophages in the lymphoid areas. In lymph nodes, the IH1 MAb labeled macrophages in the marginal sinus, some dispersed macrophages in the paracortical area, and in the medullar cords (Fig. 3). In the liver, the IH1 MAb detected cells scattered on the parenchyma. These cells showed the morphology of Kupffer cells (Fig. 2).

The IH1 antibody also stained, by indirect immunofluorescence, peripheral blood monocyte-derived macrophages in cell cultures in chamber slides. These cells, approximately on day 8 of culture, became large, round or spindle-shaped adherent cells with long dendritic projections (Fig. 4). It did not stain, by indirect immunofluorescence, human PBMC.

Cell population in peripheral blood expressing IH1 antigen

Canine cell populations from whole blood were previously identified by size (FSC) and granularity (SSC), using flow cytometric analysis, as granulocytes, monocytes and lymphocytes. The IH1 MAb was able to identify monocytic cell populations, but did not show any reactivity with granulocytes or lymphocytes. The relative percentage of marked monocytes by IH1 MAb was 86.3% (Fig. 5).

Double labeling of canine blood leukocytes was performed in order to access the reactivity of the IH1 MAb against $CD4^+$ and $CD8^+$ cells. The IH1 MAb reacted with 49.9% of $CD4^+$ and 38.8% of $CD8^+$ monocytes (Fig. 6).

DISCUSSION

The IH1 MAb, described herein, recognizes some populations of monocytes/macrophages. Under non-reducing condi-

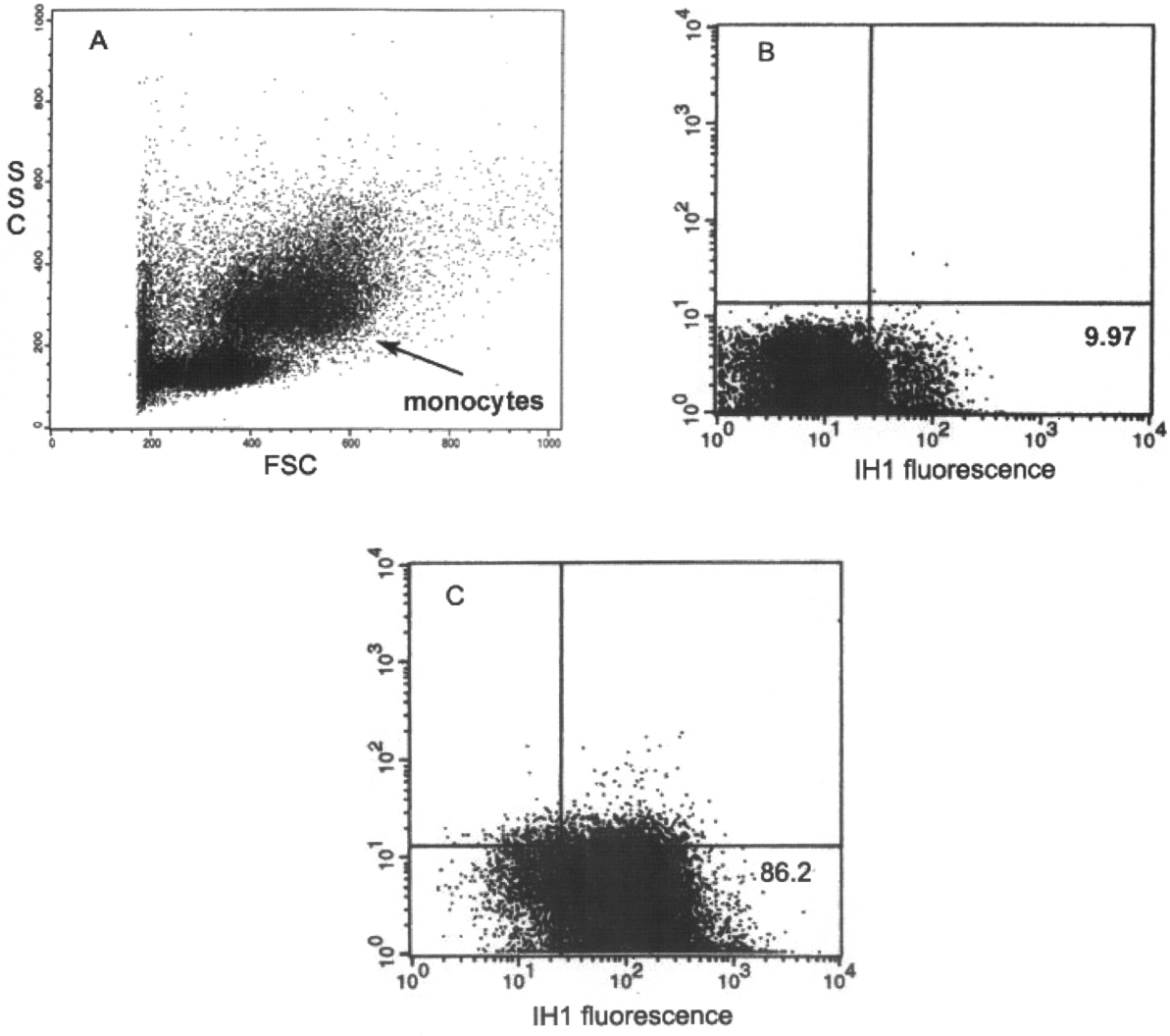

FIG. 5. Flow cytometry analysis of canine whole-blood cells. Light forward-scatter (FSC) and side-scatter (SSC) (**A**); the IH1 MAb was able to identify cell populations of the peripheral blood cells but did not show any reactivity with granulocytes or lymphocytes (**B**); relative percentage of marked monocytes by IH1 MAb (**C**). Numbers indicate the percentage of cells within the respective regions.

tions, the MAb recognized a broad protein band spread out between 55 and 73 kDa, in Western blotting using canine leukocyte lisate. This characterizes the MAb as an antibody recognizing conformational epitopes of a monocyte/macrophage antigen.

The IH1 MAb stained monocytes in PBMC, peripheral blood monocyte-derived macrophages and mature macrophages in cell culture, by indirect immunofluorescence, recognizing, therefore, an epitope expressed at early and late stages of macrophage differentiation.

A considerable number of monoclonal antibodies, which recognize human monocytes/macrophages, have been reported. However, most of them were cross-reactive with other cells. For example, anti-CD11, CD13, CD16, CD35, CD68, CD87, CD89, CD93, CD101, CD121, CD123, CD131, and anti-CD162 were found also to react with granulocytes. Anti-CD31, CD32, CD36, CD40, CD49, CD63, CD74, CD80, CD85, CD86, CD119, and anti-CD121 also labeled platelets and B cells. Others, such as anti-CD49 and CD69, labeled T cells (7^{Th} Human Leukocyte Typing Workshop).[18]

The IH1 MAb, however, recognized only cells of the monocyte/macrophage lineage and megakaryocytes: reacted with macrophages in the marginal sinus and with some dispersed macrophages in the paracortical area and in the medullar cords in the lymph node. It stained macrophages in the mantle zone and some scattered macrophages in the red pulp, as well as megakaryocytes in the spleen, and liver Kupffer cells. It did not, however, stain all monocytes: in cytometric studies, the relative percentage of monocytes stained by IH1 MAb was 86.3%. Granulocytes and lymphocytes remained negative. With flow cytometric analysis of whole blood preparations it is possible to completely discriminate canine monocytes and lymphocytes by FSC (forward-scatter, cell size) and SSC (side-scatter, cell granularity). In the monocyte subset, the IH1 MAb reacted with 49.9% of $CD4^+$ and 38.8% of $CD8^+$ monocytes.

The IH1 antibody is comparable to the antibodies that de-

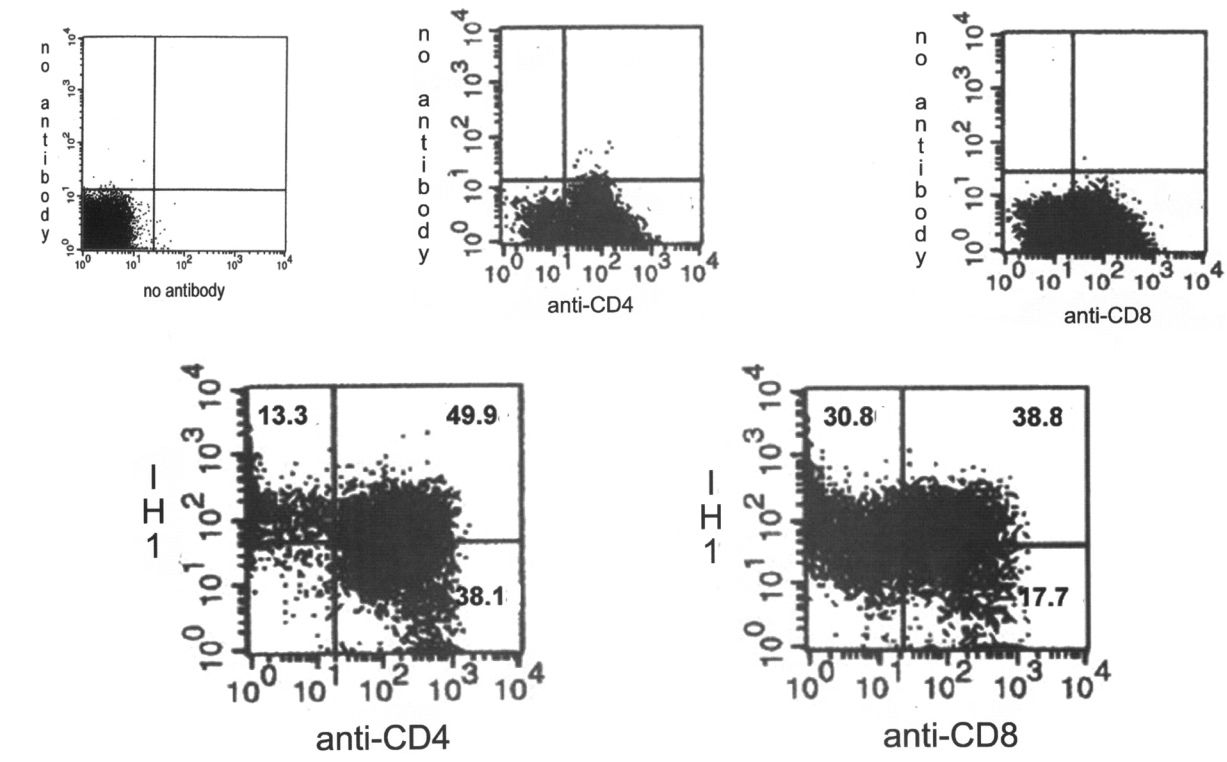

FIG. 6. Two-color flow cytometric analysis of canine whole-blood cells stained with the IH1 monoclonal antibody and anti-CD4 or anti-CD8 monoclonal antibodies. Numbers indicate the percentage of cells within the respective regions.

tected the human CD14 and CD163 antigens, concerning their cell distribution.[19,20]

The CD163 is a membrane glycoprotein with molecular weight ranging from 110 to 130 kDa, expressed on human monocytes and macrophages, in the intermediate and late phase of inflammation. Although there are similarities in the pattern of cell type and tissue distribution of the human monoclonal antibody-identified CD163, this molecule is described with a molecular weight of 110–130 kDa.[20] On the other hand, the IH1 MAb identified antigens with quite a different range of molecular weight, from 55 to 73 kDa, even though its staining pattern follows the same one described by the anti-human CD163.

A major common molecule in cells of the monocyte/macrophage lineage is the CD14 antigen, the receptor for LPS, with molecular weight of 55 kDa, and a predominant distribution on human monocytes and a variety of types of tissue macrophages.[19] A possible reactivity of the IH1 antibody with this molecule has to be investigated in further studies.

The IH1 MAb did not cross-react with human PBMC, despite some authors report that MAbs developed against human leukocytes reacted with epitopes on canine cells at a relatively high proportion, suggesting that the recognized antigens may be conserved among philogenetically distant animals.[6,7,21–23]

Among the different techniques used to identify macrophages in tissue samples, immunohistochemical labeling with monoclonal antibodies is one of the most reliable.

The routine processing of tissues for histopathological examination usually involves an aldehyde fixation step and results in a loss or decrease of antigenicity because the cross-linking of reactive sites on proteins makes certain epitopes inaccessible to antibodies. The formalin-fixed, paraffin-embedded tissues, however, have the advantage over frozen sections of easier handling and better preservation of tissue samples, improving morphologic resolution.[24]

Many monoclonal anti-leukocyte antibodies are only useful for staining frozen tissue sections with its inherent poor morphology definition, and are not readily adapted to formaldehyde-fixed and paraffin-embedded tissue.[25–27] Conventional processing of the tissue was sufficient for optimal immunohistochemical detection of IH1-antigen expressing cells. The staining pattern of the MAb was similar comparing the two techniques, immunohistochemistry and immunofluorescence, but morphologic resolution was better on formalin-fixed, paraffin-embedded tissue. Hence, the IH1 MAb constitutes a useful tool for detection of monocytes/macrophages in routinely processed paraffin-embedded tissue sections. It could allow new and retrospective studies using material commonly processed for histopathological diagnosis.

The canine immune system is of veterinary and general biological interest. The development of monoclonal antibodies against canine cluster differentiation (caCD) antigens has facilitated its characterization. Some monoclonal antibodies against canine myeloid antigens have been reported, but MAbs that exhibit an exclusive reactivity with canine monocytes/macrophages are rare.

A clearest identity of the reactivity of the MAb described herein with any antibody already reported could not be found. The assignment of an anti-CD specificity to this MAb by the identification of the recognized antigens through their amino acid sequencing is a necessary future. The IH1 MAb was shown

to be useful for detection of monocytes/macrophages in routinely processed paraffin-embedded tissue sections. Its application in immunohistochemical and flow cytometric analysis may provide valuable information on the role of macrophages in the pathogenesis of different diseases, in which these cells may be involved.

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