Production of Monoclonal Antibodies Against Canine Leukocytes

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ABSTRACT

A panel of anti-canine leukocyte monoclonal antibodies (MAbs) was produced by immunizing BALB/c mice with canine peripheral blood mononuclear cells (PBMC), either resting or stimulated with concanavalin A (ConA). Three out of 28 clones—IH1, AB6, and HG6–screened by ELISA and producing antibody with the highest specificity for canine cell immunostaining, were subjected to three subsequent subcloning steps by limiting dilution, and selected for further characterization. These MAbs belonged to IgG1 (HG6 and IH1) and IgG2a (AB6) isotypes. The distribution of cell populations expressing the antigen recognized by the antibodies was identified by indirect immunoflorescence on canine PBMC and on tissue sections of lymph node, spleen, liver and skin. The possible crossreactivity with human PBMC was also examined in immunocytochemistry. One of the antibodies specifically recognized macrophages. The MAbs presented here can be foreseen as possible valuable diagnostic and research tools to study immune functions in dogs.

INTRODUCTION

THERE IS A GREAT DEMAND for reagents to identify and characterize the canine immune response. In many human diseases that also occur in the dog, such as allergic asthma,[1] penfigus, systemic lupus eritematosus, congenital or acquired immunodeficiency[2] and in zoonosis such as leishmaniasis,[3] immunological mechanisms play an important or central role in the genesis of the lesions and the progression of disease. Moreover, the accurate diagnosis of lymphoproliferative diseases such as lymphomas and leukemias,[4,5] common in dogs, through the characterization of cell types and differentiation stages, is fundamental for treatment and definitions about the prognosis of the disease.

Dogs are also used in transplantation studies which require immunosuppressive intervention and as models for investigation of human diseases.[2] Nevertheless, little is known about the dog immune system, and canine leukocyte markers are so far only poorly characterized. For example, high expression of

CD4 has been demonstrated in canine neutrophils, a condition that has not been observed in other species.[6] There is, however, only a limited panel of available canine cell markers and this lack of reagents has impaired the development of studies on canine leukocyte pathology.[6–11]

In the present study, the production of 28 new monoclonal antibodies (MAbs) directed against canine leukocytes is reported. Three of these antibodies were selected for characterization, in order to build a panel of monoclonal antibodies against canine leukocytes with possible use for research and diagnosis of canine diseases.

MATERIALS AND METHODS

Animals and tissues

Two normal outbred dogs, with estimated ages of 24–36 months, with 15 kg of weight, were used throughout the study.

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The dogs were kept separately, under standard care and feeding conditions, in the kennel of Gonçalo Moniz Research Center, Oswaldo Cruz Foundation and used as blood donors. Normal dog tissues were from the Department of Pathology and Clinics, Veterinary School, Federal University of Bahia, and derived from necropsy material. Ten 6-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.

Canine and human peripheral blood mononuclear cells (PBMC)

Blood was collected by venipuncture of the cephalic vein into tubes containing enough heparin to produce a final concentration of 10 IU mL^{-1}. The peripheral blood mononuclear cells (PBMC) were isolated by centrifugation of diluted blood layered on Histopaque 1077 (Sigma Chemical Co., St. Louis, MO). Cells were washed twice in HBSS (Hank's balanced salt solution, Sigma Chemical Co.) before being used in the different steps.

Antigen preparation

Mitogen stimulated PBMC were obtained by incubating these cells in the concentration of 5×10^6/mL, with 5μg of concanavalin A (ConA, Sigma Chemical Co.) in RPMI 1640 (Sigma Chemical Co.) supplemented with 100 IU mL^{-1} of penicillin, 100 μg mL^{-1} of streptomycin, 2 mM L-glutamine and 20% of heat inactivated fetal calf serum (GIBCO, MD, USA), for 48 h in a 100% humidified atmosphere containing 5% CO_2 at 37°C. Stimulated and non-stimulated canine PBMC were adjusted for 10^7 cells/mL and sonicated before emulsification with Freund's adjuvant.

Immunization

Groups of five BALB/c mice were immunized with ConA-stimulated or non-stimulated canine PBMC, sonicated and emulsified with an equal volume of complete Freund's adjuvant (Sigma Chemical Co.). The animals were injected with an amount of antigen equivalent to 10^7 cells per dose, one injection via intraperitoneal and two subcutaneous injections. Two identical boosters were given two and four weeks after the first immunization, using the same dose regimen, emulsified in incomplete Freund's adjuvant (Sigma Chemical Co.). Two weeks after the second booster, the mice were tested for the presence of circulating antibody against canine PBMC, by indirect ELISA. The animal with the highest antibody titer was injected intravenously with 10^7 canine PBMC, lysed by sonication, in 200 μL of 0.15M PBS, pH 7.2.

Fusion

Three days after the final booster, the animal was sacrificed and a single-cell splenocyte suspension was prepared and fused with the SPO_2 myeloma cells, following standard procedures.[12] After fusion, cells were distributed in ten 96-well tissue culture plates with RPMI supplemented with 100 IU mL^{-1} of penicillin, 100 μg mL^{-1} of streptomycin, 2 mM L-glutamine

and 10% of heat inactivated fetal calf serum, supplemented with HAT (100mM hypoxantine, 0.4 μM aminopterine and 16 μM thymidine). After 14–21 days, hybrid cells showing antibody activity against canine leukocyte screened by ELISA, were cultured further using a layer of feeder cells from a nonimmunized BALB/c mouse to assist in the early stages of growth. Antibody-producing cells were subjected to three subsequent rounds of subcloning steps, by limiting dilution, for obtaining stable hybridomas. Culture supernatants were used in subsequent experiments.

Cellular indirect enzyme-linked immunosorbent assay (ELISA)

Canine PBMC were adjusted to the concentration of 5×10^5/mL in RPMI medium and 100 μL of the cell suspension delivered into wells of a 96-well microplate (Nunc, Naperville, IL). The plate was centrifuged for 5 min at 700g, at 4°C. After carefully discarding the supernatants with a multichannel pipette, the cells were air-dried at 37°C for at least 1 h and fixed for 5 min with 200 μL of 100% ethanol. The plate was washed with PBS and the endogenous peroxidase was blocked with PBS containing 1% H_2O_2 for 1 h at room temperature (RT). Remaining binding sites were blocked with 150 μL of PBS containing 5% skimmed milk powder (w/v), for 1 h at RT. MAb supernatants were tested at 1:2 dilution in PBS containing 0.05% Tween-20 (v/v), 5% skimmed milk powder (w/v) and 10 μg/mL of rat IgG. After 1 h incubation at RT, the supernatants were removed, the plate was washed with PBS containing 0,05% Tween-20 (PBS-Tween), and a peroxidase-conjugated polyclonal sheep anti-mouse IgG (Sigma Chemical Co.), diluted 1:1000, was added. After 1 h of incubation at RT the unbound conjugate was removed through washings with PBS-Tween. The reaction was revealed with 5 mg/mL of orto-phenylenediamine (OPD, Sigma Chemical Co.) in citrate-phosphate buffer and stopped with 20 μL of 4N H_2SO_4. Optical densities were read at 490 nm.[13]

Isotyping of the monoclonal antibodies

The isotypes of MAbs were determined 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 a protocol recommended by the manufacturer.

Western blotting

PBMC (10^7 cells) were lysed in SDS-PAGE sample buffer, pH 6.8 (Tris-HCl 62.5 mM, 2% (w/v) SDS and 10% (v/v) glycerol), under reducing (with 2-mercaptoethanol) and non-reducing (without 2-mercaptoethanol) conditions. The samples were loaded onto 0.7 mm 10% SDS (w/v) polyacrilamide slab gels, and electrophoresis performed as described elsewhere.[11] Proteins were then elctrophoretically transferred to nitrocellulose membrane and subjected to Western blotting against the hybridoma supernatants diluted 1:2 with PBS-Tween containing 5% skimmed milk powder (w/v; PBS-T20-milk) as described previously, using peroxidase-conjugated sheep anti-mouse immunoglobulin polyclonal antibodies (Sigma Chemical Co.) and a mixture of 3,3'-diaminobenzidine (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.

Immunoprecipitation

Canine spleen cells were suspended in PBS (pH 7.2) and subjected 12 times to sonification with bursts of 20 sec (Sonigier 450/Brandson) and treated with 100 mM NaCl and 1 mM PMSF for 1 h at 4°C. Insoluble fractions were removed by microcentrifugation at 15000 rpm for 30 min. Before immunopreciptation, MAbs were coupled to protein A-sepharose beads (Pharmacia, Uppsala, Sweden). The preclearation of all the spleen cell extracts was made with 1 mL of proteins for 1 h with 100 μL of protein A-sepharose beads. Then the mixture was incubated with 100 μl of each MAb-coated sepharose beads for 1 h at 4°C with continuous rotation. The beads were washed with PBS-T, resuspended with sample buffer and subjected to SDS-PAGE. The bands corresponding to the molecules recognized by the MAbs were visualized by western blotting.

Indirect immunofluorescence

Immunofluorescence reactions were preceded on permeabilized or non-permeabilized canine and human PBMC with Tween-20. Also, cryostat sections (6–8 μm in thickness) of canine normal lymph node, spleen, liver, skin, and kidney were carried out and tested by indirect immunofluorescence. PBMC or tissue sections were air-dried and fixed in alcohol for 5 minutes at 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-Tween (0,05%) with skimmed milk. The detergent was used for membrane permeabilization to guarantee the access of the MAbs to cytoplasm epitopes. Irrelevant antibody, diluent and normal mouse serum (diluted 1:500) were used as negative controls. The wells were washed three times in PBS and incubated for 30 min with secondary fluoresceine isothiocyanate (FITC)–conjugated sheep IgG anti-mouse immunoglobulins (Sigma Chemical Co.) diluted 1:200 in Blue Evans working solution. The slides were then washed three times with PBS for 10 minutes mounted with glycerol buffer and examined by UV microscopy.

Production of isotype control immunoglobulins

Normal mouse immunoglobulins with the same isotypes of the MAbs were used as controls for monoclonal antibody activity. A globulin-rich fraction was prepared from sera obtained from healthy female BALB/c mice by precipitation with 40% saturated ammonium sulphate. After dialysis against PBS pH 8, mouse IgG1, IgG2a, and IgG2b immunoglobulins were purified from the globulin fraction by affinity chromatography on protein A–Sepharose 4B (Amersham Pharmacia Biotech, Uppsala, Sweden) with immunoglobulins isotype elution as a function of pH.[14] Briefly, when the globulin fraction was applied on the column at pH 8.0, IgM, IgA, and IgE were almost quantitatively recovered in the effluent, together with non-immunoglobulin serum components and essentially all IgG was retained at the column. Using buffers of decreasing pH, IgG1, IgG2a, and IgG2b were sequentially eluted at pH 6.0–7.0, pH 4.5–5.0 and pH 3.5–4.0, respectively. The eluted immunoglobulins were tested for isotype and stored at −20°C.

Macrophage differentiation in vitro

Canine PBMC were adjusted to the concentration of 5×10^6/mL in RPMI medium supplemented with 20% of heat inactivated fetal calf serum and 500 μL of the cell suspension were delivered into flat-bottomed six-well culture plates (Nunc, Naperville, IL), carefully washed after 24 h and incubated for 5–10 days in a humidified atmosphere at 37°C and 5% CO_2. Canine macrophages, differentiated from peripheral blood monocytes, adhered to the well bottoms and were used in indirect immunofluorescence tests using the MAbs and control immunoglobulins as described above.

RESULTS

Twenty-eight clones obtained from the fusions recognized canine leukocyte antigens in ELISA, yielding three stabilized hybridomas, with different specificities, named AB6, IH1 and HG6, which were selected for further characterization.

The isotyping of the three studied hybridomas revealed that the AB6 produced an IgG2a monoclonal antibody; and HG6 and IH1 produced monoclonal antibodies of the IgG1 subclass.

Data on the staining pattern of canine PBMC by immunocytochemistry and on the molecular weights of the antigens recognized by the MAbs in Western blotting analysis are summarized in Table 1. Under non-reducing conditions, the AB6 and HG6 MAbs detected single protein bands with molecular weights of 185 and 46 kDa, respectively. The IH1 MAb recognized a broad protein band spread out between 76 and 45 kDa (Fig. 1). Under reducing conditions, no antigenic band was detected by any of these antibodies. We further investigated whether the MAbs were useful for immunoprecipitation of canine leukocyte molecules. Canine spleen cell extracts were in-

TABLE 1. MONOCLONAL ANTIBODY ISOTYPES, MOLECULAR CHARACTERIZATION, IMMUNOLOGICAL CROSS-REACTION ON THE HUMAN PBMC BY INDIRECT IMMUNOFLUORESCENCE AND STAINING PATTERN OF THE CANINE PBMC-SPECIFIC MONOCLONAL ANTIBODIES

| | | | Reaction | Canine PBMC | |
MAbs	Isotypes	MW (kDa)	human-PBMC	Population	Staining pattern
AB6	IgG2a	185	Neg	LY, PMNN	Diffuse—membrane and cytoplasm
HG6	IgG1	46	Neg	LY	Diffuse—membrane and cytoplasm
IH1	IgG1	73–45	Neg	LY, Mϕ	Focal—membrane and cytoplasm

neg, negative; Ly, lymphocytes; PMNN, polymorphonuclears; Mϕ, macrophages.
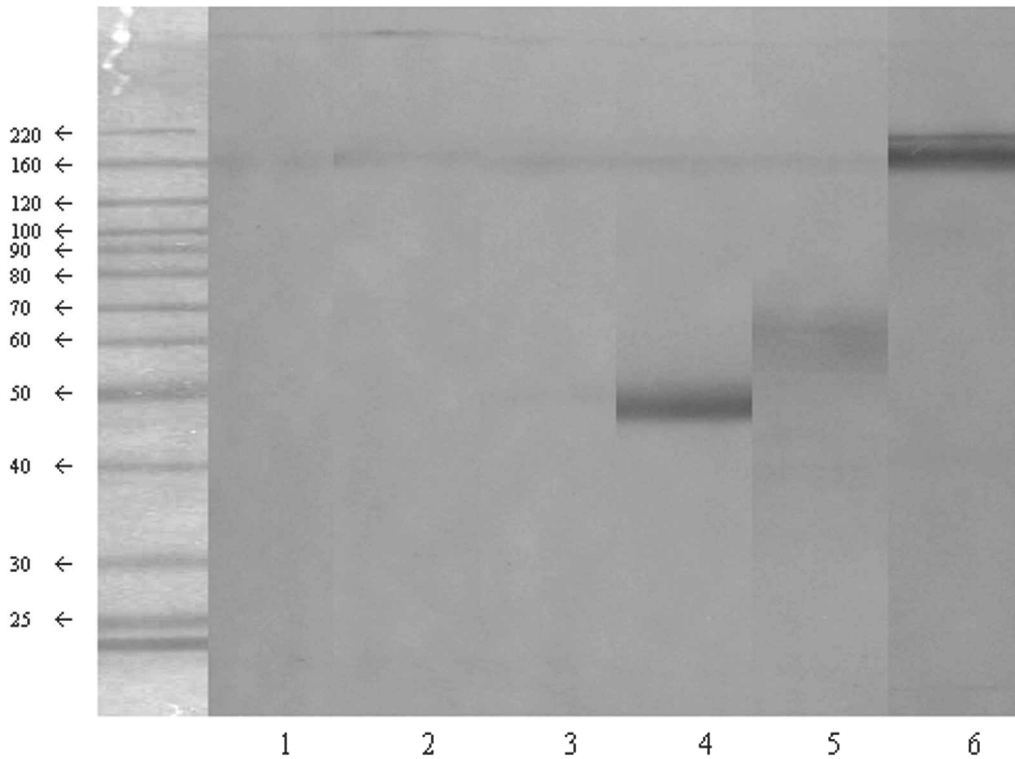
FIG. 1.   Western blot of the cell lysates of canine PBMC in nitrocellulose membrane after transferring from non-denaturating SDS-PAGE showing reactivity of MAbs and controls: saline (1); normal mouse serum (2); anti-*Leishmania* MAb (3); HG6 MAb (4); IH1 MAb (5); and Ab6 MAb (6). On the left side is the molecular weight pattern stained in Comassie Blue to show the relative molecular weight of the recognized proteins by the MAbs.

cubated with protein A–sepharose beads coated with each MAb and the immunoprecipitates were visualized by western blot analyses using the MAbs AB6, HG6, and IH1.

The AB6, HG6, and IH1 MAbs stained cells with and without membrane permeabilization with Tween-20. The IH1 MAb stained a small population of PBMC cells with morphologic characteristics of monocytes, and also stained *in vitro* peripheral blood monocyte-derived macrophages (Fig. 2).

The three MAbs stained the cell membrane. The AB6 and HG6 MAbs showed a diffuse, homogeneous pattern of staining, while IH1 showed a multi-focal pattern on the cell surface (Table 1).

The three MAbs were tested in tissue preparations: IH1, AB6, and HG6. The IH1 proved its specificity for macrophages, showing a characteristic staining pattern in the spleen, lymph node and liver, in which the Kuppfer cells were stained. The other antibodies (AB6 and HG6) marked mononuclear cells in
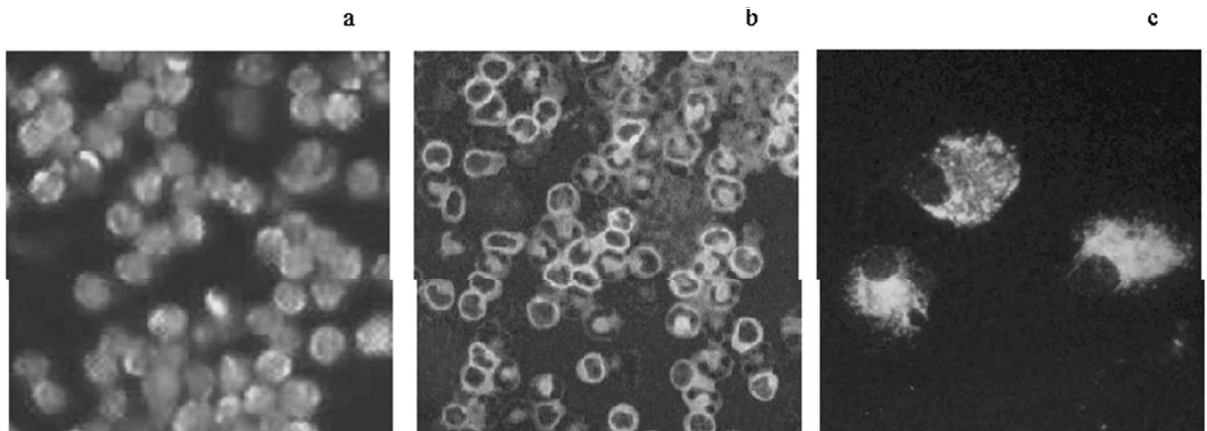

FIG. 2.   Immunofluorescent staining of canine cells with MAbs: (**a**) Preparation of PBMC in suspension, stained with HG6 MAb showing a pan-leukocyte identification. (**b**) Preparation of PBMC by cito-centrifugation followed by acetone fixation and staining with AB6 MAb diluted in Blue Evans, identifying a lymphocyte subset. (**c**) Canine macrophages differentiated from a peripheral blood monocytes culture, stained by the IH1 MAb. ×400.

spleen and lymph node. HG6 stained only large mononuclear and not reticular cells, in the germinative center of the follicle. AB6 stained a large population of mononuclear cells, including interstitial cells. In liver, HG6 stained Kuppfer cells, while AB6 marked only round lymphocyte-like cells, on small inflammatory foci in parenchyma. On the skin, AB6 and HG6 stained cells in inflammatory infiltrate foci, while IH1 and HG6 stained spindle cells, some of them with irregular projections, present in the edge of the hair follicles (Fig. 3). None of these MAbs cross-reacted with human PBMC (Table 1).

DISCUSSION

In this report, we present three monoclonal antibodies raised against canine leukocytes, including one recognizing macrophages. Under non-reducing conditions, all the MAbs identified different sharp single protein bands, varying from 185 to 45 kDa, demonstrating their different specificities. The IH1 MAb recognized a broad protein band, spread out between 76 and 45 kDa, similar to a glycosylated protein pattern. None of them show any reaction under reducing conditions, indicating that they may recognize conformational epitopes. The analyses of their reactivity against PBMC demonstrated that all were capable of identifying membrane and/or cytoplasm proteins, and none reacted only with cytoplasm proteins. Hence, the MAbs can possibly be used to identify specific caCDs or to modulate canine immune responses. Moreover, these MAbs recognized a variable pattern of epitope distributions on the cells, which could be classified as diffuse, and could indicate different roles on recognizing molecules in cell function.

The IH1 MAb identified monocyte/macrophage cells on the different tissues studied. Only a very small number of reported MAbs exhibit an exclusive reactivity with monocytes/macrophages.[15,16] None of these, however, have the same specificity as the IH1 MAb, which reacted with monocytes on peripheral blood, mature macrophages in cell cultures and different macrophage subpopulations in tissues, such as spleen cortical and red pulp, liver Kupffer cells and Langerhan's type cells in the skin.

The staining pattern produced by the HG6 MAb suggest that it identifies phagocytic leukocytes, while the pattern produced by the AB6 indicates that it recognizes lymphocytes.

In order to evaluate the ability to recognize leukocyte antigens from other species, the selected MAbs were assayed by immunofluorescence using human PBMC as targets. None of them identified epitopes on human leukocytes. It was reported that reactions of MAbs developed against human leukocytes with epitopes on canine cells happened at a relatively high proportion, suggesting that the recognized antigens may be conserved among phylogenetically distant animals.[17] In fact, 10% of cross-reactions were found by Joachin et al.[18] using monoclonal antibodies, between leukocytes from different species, such as dog and cattle. However, although in some cases, MAbs directed against antigens from one species recognize leukocytes of other species, the majority of MAbs do not cross-react.[6,8,15,19]

A more extensive determination of the properties of the MAbs described herein would require further studies. The identification of the recognized antigens through their amino acid
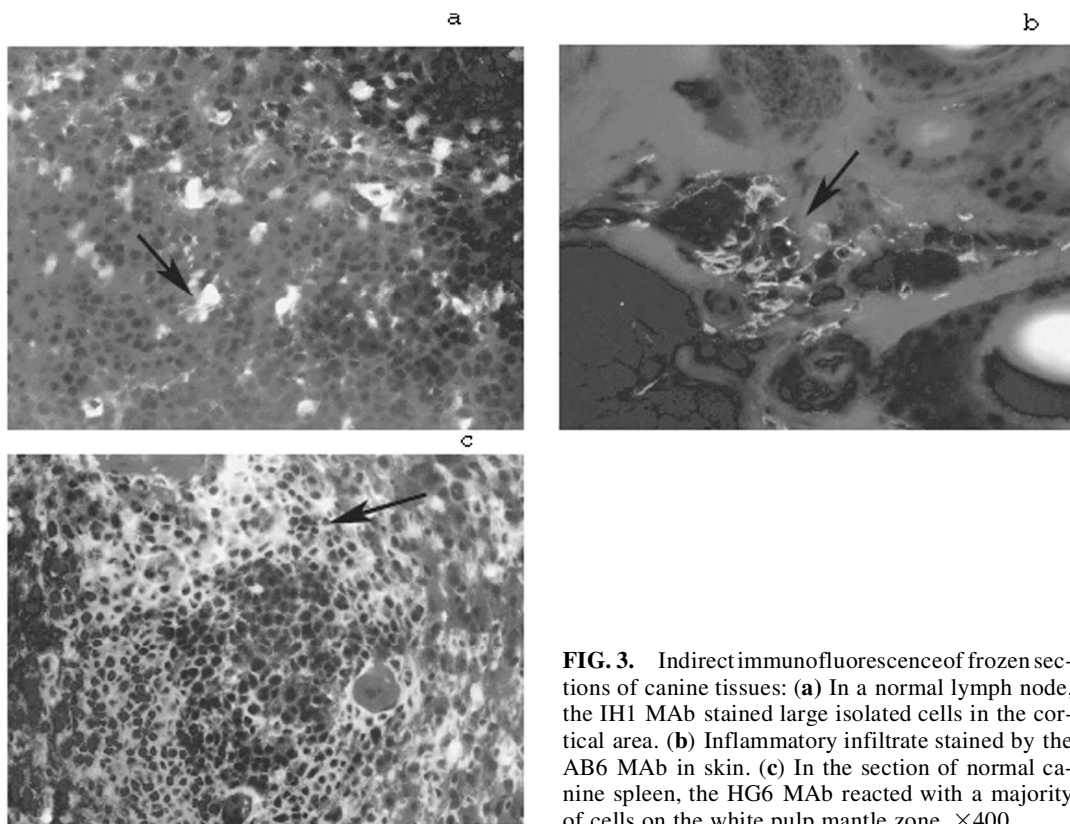
FIG. 3. Indirect immunofluorescence of frozen sections of canine tissues: (**a**) In a normal lymph node, the IH1 MAb stained large isolated cells in the cortical area. (**b**) Inflammatory infiltrate stained by the AB6 MAb in skin. (**c**) In the section of normal canine spleen, the HG6 MAb reacted with a majority of cells on the white pulp mantle zone. ×400.

sequencing would allow their use in immunotherapeutic studies of canine diseases in which the immune system plays a pathogenic role; in immunoassay-based diagnoses of lymphomas and leukemias, and in achieving a fuller understanding of the canine immune response in infection and autoimmunity.

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