Production of a Monoclonal Antibody (59.4) against Canine Lymphocyte Surface Antigen and Its Immunohistochemical Application

Yukihiko IWAMI, Yoshiharu HASHIMOTO, and Toshihiko IWANAGA

Laboratory of Anatomy, Department of Biomedical Science, Graduate School of Veterinary Medicine, Hokkaido University, Kita 18-Nishi 9, Kita-ku, Sapporo 060, Japan

(Received 31 October 1996/Accepted 17 December 1996)

ABSTRACT. A monoclonal antibody was produced by immunizing BALB/c mice with freshly prepared canine thymocytes and peripheral blood leukocytes. The antibody, designated 59.4, was of the IgG1 subclass type and mainly reacted with lymphocytes. In single-color flow cytometric analysis, lymphocytes from the peripheral blood, thymus and spleen were graded into three categories according to their fluorescence intensity labeling by antibody 59.4: weakly, moderately and intensely positive cells. Two-color analysis revealed that a major population of CD8-positive cells were intensely labeled by antibody 59.4, but less than 50% of CD4-positive cells were moderately reacted with antibody 59.4. Immunohistochemically, thymocytes in the medulla showed moderately intense immunoreactivity to 59.4, but most lymphocytes in the cortex were negative in reaction. Immunostaining using antibody 59.4 demonstrated characteristic aggregations of 59.4-positive lymphocytes in the reticulum cell-free region of the thymic medulla. In the spleen, scattered lymphocytes in the outer layer of the marginal zone and in the red pulp were intensely labeled by antibody 59.4, while lymphocytes gathering in the mantle zone and periarterial lymphatic sheath (PALS) were moderately stained. Antibody 59.4 appears to recognize an antigen which is expressed by a more-differentiated T cell-lineage but not by immature T cells in the thymic cortex. — KEY WORDS: canine, flow cytometry, immunohistochemistry, monoclonal antibody, thymus.

J. Vet. Med. Sci. 59(4): 239–244, 1997

Monoclonal antibodies against leukocyte surface antigens have been used extensively for identification of various types of immune cells in the field of veterinary medicine [9, 12, 14]. Numerous monoclonal antibodies have also been raised against canine leukocytes, as reported in an international workshop on canine leukocyte surface antigen [2]. However, few antibodies are available for both flow cytometric and histological analysis of immune cells in the dog.

We have produced novel monoclonal antibodies against canine leukocytes to investigate their distribution, origin and functional significance by flow cytometry and immunohistochemistry. The monoclonal antibody (59.4) presented here reacted mainly with a subtype of lymphocytes in peripheral blood and some lymphoid organs. Immunohistochemical application of the antibody in the thymus revealed the presence of unknown lymphocyte foci which are structurally different from the thymic parenchyme.

MATERIALS AND METHODS

Animals, immunization schedule and cell fusion: BALB/c mice, originally purchased from Nippon CLEA (Tokyo, Japan), and mongrel dogs were raised in our breeding facilities.

Two mice were immunized with a subcutaneous injection of an emulsion consisting of Freund's complete adjuvant and 2×10^7 thymocytes freshly prepared from a 2-month-old puppy. At 16 weeks after the initial immunization, the mice were boosted intravenously and intraperitoneally with 2×10^6 leukocytes obtained from the peripheral blood of another dog 4 years old. The canine peripheral blood leukocytes (PBL) were isolated from the buffy coat and treated twice with a red cell-lysis buffer (140 mM NH_4Cl in 17 mM Tris-HCl buffer, pH 7.4) to remove platelets and erythrocytes. Four days after the boost, mouse spleens were prepared aseptically for cell fusion. Fusion of the mouse spleen cells and myeloma P3X63 Ag 8-U1 was performed according to the general procedure described by Köhler and Milstein [8].

Screening assay, cloning and isotype analysis: Hybridoma supernatants were tested primarily by flow cytometric (FCM) analysis. The procedure, including the selection of buffers for indirect immunofluorescence labeling, was according to a protocol recommended by the Canine Leukocyte Antigen Workshop (CLAW) [2]. Culture supernatant (50 μl) was incubated for 45 min with 1×10^6 PBL of dogs. After the cells were washed three times, they were incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse immunoglobulins (Organon Technica Corp., U.S.A.) diluted 1:100 for 45 min, and then analyzed with an Epics Profile (Coulter, U.S.A.) after three washing steps and formaldehyde fixation.

Some of the hybridoma supernatants, which exhibited different histogram patterns for lymphoid and non-lymphoid cells in the primary FCM analysis, were subsequently screened on tissue sections using the peroxidase-anti-peroxidase (PAP) method. Lymphoid organs of a 2-month-old puppy and a 1-year-old dog were used for the immunohistochemical examination. The thymus, lymph nodes, spleen and palatine tonsils were removed and snap-frozen in liquid nitrogen. Cryostat sections, 6 μm-thick, were air-dried and fixed with ice-cold acetone for 5 min. They were incubated overnight with the undiluted

hybridoma supernatants or normal mouse serum diluted 1:100 with 0.01 M phosphate-buffered saline (PBS, pH 7.4) as a negative control.

An antibody-producing hybridoma, showing intense reactivity with lymphocytes in FCM analysis and immunostaining, was recloned twice by limiting dilution and denominated 59.4. Immunoglobulin classes and subclasses of the antibody were determined by means of a mouse monoclonal antibody isotyping kit (Amersham, England).

Characterization of monoclonal antibody 59.4:

(1) *Additional FCM analysis*: FCM analysis showed that the peripheral blood contained both lymphocytes and non-lymphoid cells reactive with antibody 59.4. To characterize non-lymphoid cells, PBL collected from three adult mongrel dogs were resuspended, at a concentration of 2×10^6 cells per m*l*, in RPMI-1640 medium containing 10% fetal calf serum and incubated in cell culture dish (Corning, U.S.A.) for 1 hr at 37°C. Non-adherent cells were collected with PBS and subjected to FCM analysis.

PBL from three mongrel dogs, and the thymus and spleen from another two-month-old beagle were used in single- and two-color FCM analysis [2]. These samples were analyzed using antibody 59.4 (supernatant or biotinylated) and commercially available antibodies, i.e. anti-canine CD4 rat monoclonal antibody (Serotec, England), anti-canine CD8 rat monoclonal antibody (Serotec, England) and anti-canine Thy-1 mouse monoclonal antibody (Serotec, England). Immunoreactions with the 59.4 supernatant, anti-Thy-1, anti-CD4 and anti-CD8 were detected using FITC-labeled anti-mouse IgG or rat IgG goat antisera (Organon Technica Corp., U.S.A.). Biotinylation of antibody 59.4 was carried out using NHS-biotin (American Qualex, U.S.A.) according to the protocol suggested by the supplier. Reactivity with the biotinylated 59.4 was detected using B-phycoerythrin-conjugated streptavidin (Cosmo Bio, Japan).

(2) *Immunohistochemistry*: Distribution of antigens detectable with antibody 59.4 was examined on tissue sections. Fresh tissues of the thymus and spleen were obtained from four mongrel dogs, from 2 months to 3 years old. They were snap-frozen in liquid nitrogen, and serially cut in a cryostat. The cryostat sections were fixed with ice-cold ethanol and processed by the avidin-biotin-peroxidase complex (ABC) method using antibody 59.4 and the commercially available antibodies described above, and also an anti-human cytokeratin rabbit polyclonal antiserum (Progen Biotechnik GmbH, Germany).

RESULTS

Screening, cloning and isotype analysis of monoclonal antibody 59.4: Ninety-nine clones among 169 hybridomas obtained showed a positive reaction to PBL by FCM screening. Immunohistochemistry revealed that one of them (hybridoma No. 59) showed intense and characteristic stainability on lymphoid tissue sections. The monoclonal antibody was established after two-time cloning by limiting dilution and designated 59.4. In the isotype analysis, antibody 59.4 was determined to be of the mouse IgG1 subclass.

FCM analysis of plasma leukocytes and cell suspensions of lymphoid organs using antibody 59.4: Analyses of cytograms demonstrated that lymphocytes reactive with antibody 59.4 were generally divided into three populations according to the intensity of fluorescence: intensely, moderately and weakly positive ones (Fig. 1). In single-color analysis of the peripheral blood (Fig. 1a), antibody 59.4 reacted with approximately 80% of lymphocytes and 10% of non-lymphoid cells. The non-lymphoid cells showing a weak affinity to 59.4 were completely deleted after cell-culture-dish treatment, implying that they were adherent cells (Fig. 2). The former lymphocytes in the peripheral blood were classified into two populations showing intense or moderate immunoreactivities (Fig. 1a). When the peripheral blood lymphocytes were analyzed by two-color cytometry using antibody 59.4 and other commercially available antibodies, antibody 59.4 reacted with Thy-1^+ cells (data not shown). $CD8^+$ lymphocytes were largely included among those labeled intensely by 59.4 (Fig. 3a). Up to 50% of the $CD4^+$ cells showed moderately intense immunoreactivity with antibody 59.4 (Fig. 3b).

Leukocytes from the spleen and thymus were assayed by single-color analysis with antibody 59.4. Immunoreactive lymphoid cells from the spleen were divided into two populations (Fig. 1b). Those two populations showed intensely or moderately immunoreactivities as mentioned in the peripheral blood. On the other hand, the cell suspensions from the thymus consisted of two lymphocyte populations showing different intensities of immunoreaction (Fig. 1c); 15–20% of thymocytes showed moderate immunoreactivity with 59.4 while 60–70% of the cells had weakly positive reactions.

Immunohistochemistry: In the thymus, majority population of cortical thymocytes were negative to antibody 59.4 (Fig. 4a). Several cells intensely immunoreactive with 59.4 were scattered in the cortex, mainly being distributed along arterioles perpendicularly penetrating the cortex (Figs. 4a, b). The thymus medulla contained moderately immunolabeled cells. Some of them were dispersed throughout the medulla; the others formed large and compact aggregations close to the corticomedullary boundary (Fig. 5a). When adjacent sections were stained with the anti-cytokeratin antibody that recognized the epithelial reticulum cells, the lymphocyte aggregations were shown to completely lack cytokeratin-positive cells (Fig. 5b). The characteristic lymphocyte foci were round in shape and around 100-300 μm in diameter. Usually, each lobule of the thymus contained one lymphocyte focus. Almost all the lymphocytes in the foci were immunonegative with anti-CD8 antibody (Fig. 5c) but scattered lymphocytes were stained positively with anti-CD4 antibody. Neither prominent substructure, such as a germinal center, nor typical postcapillary venules were seen within the foci.

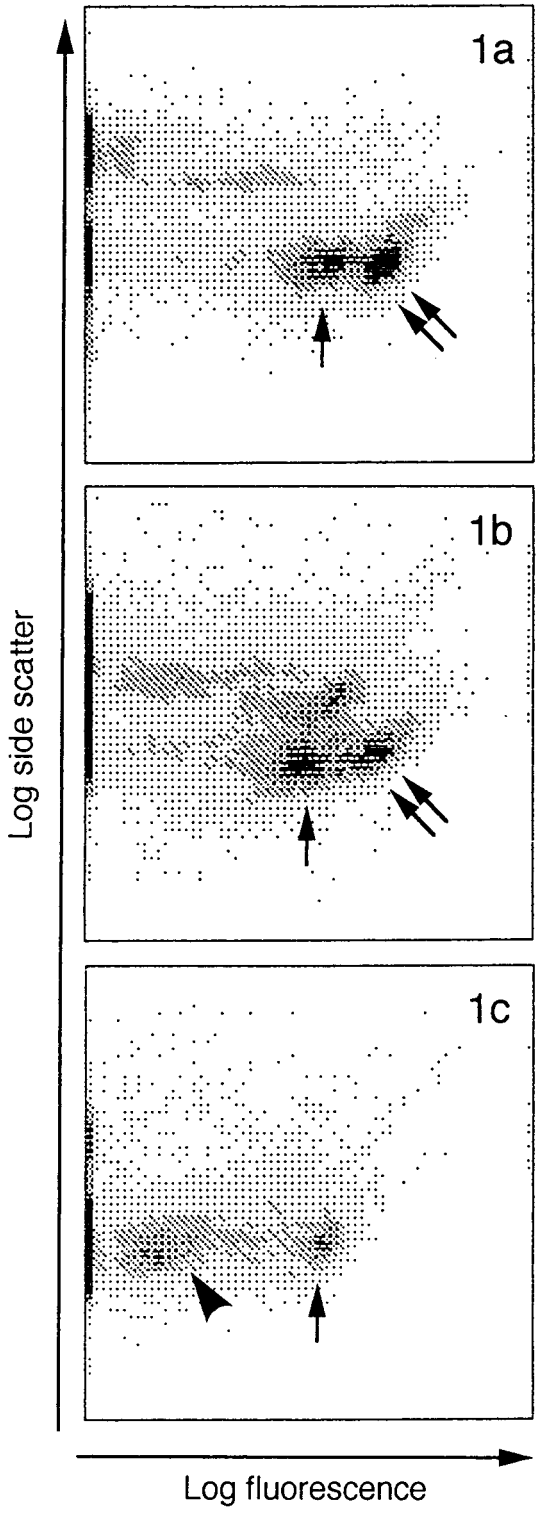

Fig. 1. Representative FCM cytograms of canine leukocytes from the peripheral blood (1a), spleen (1b) and thymus (1c) reacted with antibody 59.4. Lymphocytes are graded into intensely (double-arrows), moderately (single-arrow) and weakly (arrowhead) positive populations.

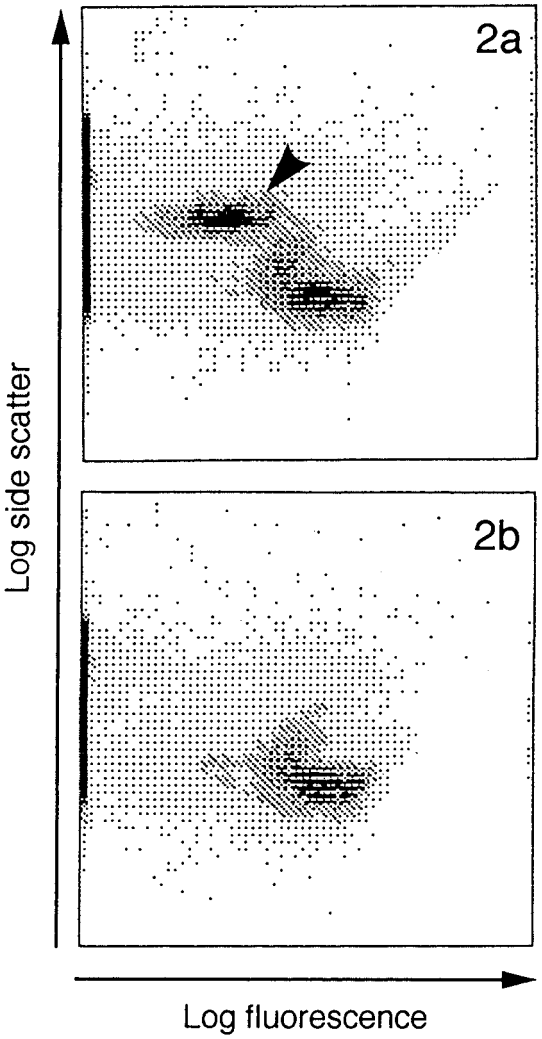

Fig. 2. Depletion of a cell population by tissue-culture-dish treatment. The non-lymphoid cell-population of the peripheral blood, weakly labeled by 59.4 (2a; arrowhead), is completely deleted after the treatment (2b).

In the spleen, antibody 59.4 strongly labeled scattered cells in outer layer of the marginal zone and some single lymphocytes in the red pulp (Figs. 6a, b). Moderately positive small lymphocytes were extensively distributed, mainly in the mantle zone and periarterial lymphatic sheath (PALS) (Fig. 6a). The germinal center itself remained almost unstained.

DISCUSSION

In the present study, a monoclonal antibody, 59.4, was raised by immunizing mice with canine thymocytes and PBL. FCM analysis using antibody 59.4 demonstrated that intensely immunopositive lymphocytes were restricted to the peripheral blood and spleen, and were not contained in the thymus. Immunohistochemically, moderately positive

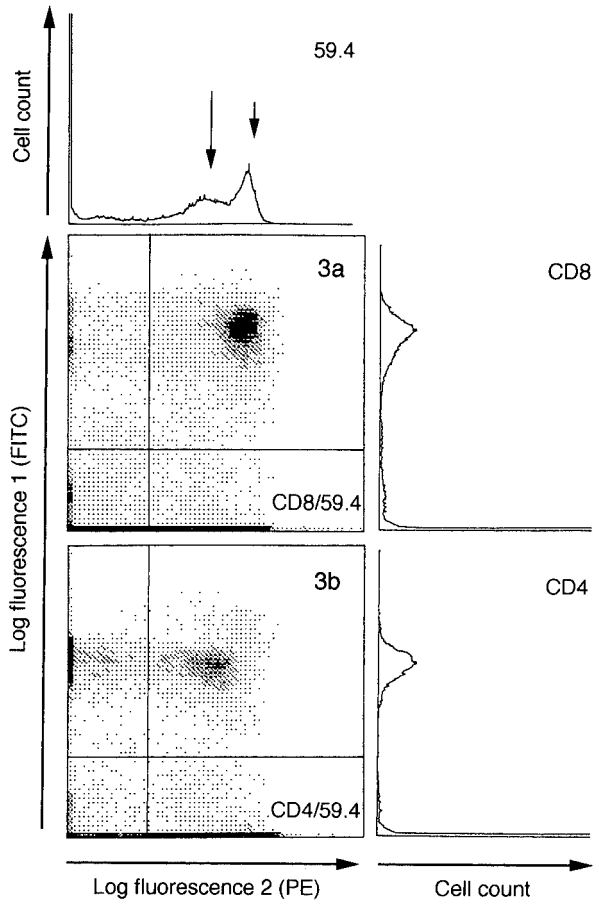

Fig. 3. Two-color FCM analysis of canine peripheral blood reacted with CD8-FITC and biotinylated 59.4-avidin-PE (3a), and CD4-FITC and biotinylated 59.4-avidin-PE (3b). Most of CD8+ cells (85%) are intensely reactive with 59.4 (59.4 histogram; short-arrow), while approximately half of CD4+ cells (53%) are moderately reactive with 59.4 (59.4 histogram; long-arrow).

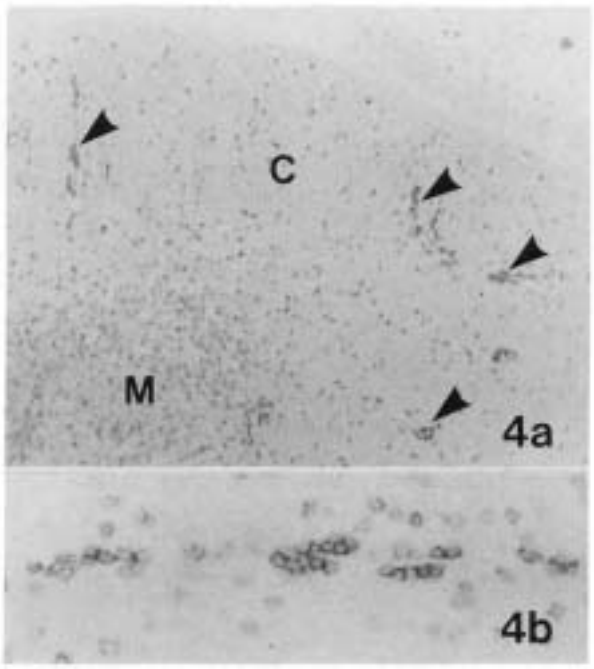

Fig. 4. Immunoperoxidase reaction of canine thymus with antibody 59.4. (4a) Moderately immunoreactive cells are distributed mainly in the medulla (M), while intensely labeled cells gather in the perivascular space (arrowheads) of the cortex (C). × 80. (4b) A higher magnification of the thymic cortex showing intensely labeled lymphocytes in the perivascular space. × 275.

thymocytes gathered in the medulla, except for some intensely positive cells scattered in perivascular spaces of the cortex. The different immunoreactivities of lymphocytes according to their location has also been recognized in cases of other lymphocyte surface antigens. Lanier *et al.* [10] reported that some lymphocyte antigens such as CD3 have increased immunoreaction intensity during T-cell maturation in humans. Thus, FCM analysis of CD3-positive cells demonstrated that cortical thymocytes, immature in nature, and medullary thymocytes, more differentiated, expressed dim and moderately bright fluorescence, respectively. On the other hand, mature T cells, e.g. peripheral blood T cells show the highest antigen density for CD3, identifiable as the brightest fluorescence [10]. Therefore, the present data obtained from flow cytometry and immunohistochemistry suggest that antibody 59.4 identifies mature or more-differentiated T cells in the peripheral blood, spleen and thymic medulla, but not immature T cells in the cortex of the thymus. The present immunostaining of the thymus demonstrated the existence of some intensely labeled lymphocytes in the perivascular spaces of the cortex. Since Kato and Schoefl [7] reported that the postcapillary venules and perivascular space in the thymus of guinea-pig may function as pathways for the migration of lymphocytes into or out of the blood circulation, it is presumed that these lymphocytes may remigrate into the thymus from peripheral blood.

Two-color flow cytometry, which allows simultaneous, multiparametric evaluation of marker substances, is useful for more detailed identification of immunoreactive cells. Antibody 59.4 could distinguish CD8+ cells from other T-cell subpopulations: most CD8+ lymphocytes intensely co-expressed 59.4 antigen, whereas some CD4+ cells expressed it moderately. These FCM findings coincided with the cellular distribution of 59.4+ cells and well-known T-cell subpopulations in the thymus and spleen. The distribution of 59.4+ cells in lymphatic organs overlapped that of Thy-1+ cells, but was not identical. Cells intensely positive for 59.4 included CD8+ cells. These findings led us to a conclusion that antibody 59.4 could detect one of the antigens expressed by mature T lymphocytes and related to CD8 antigen. The relation between the labeling intensity with 59.4 antibody and the maturation stages of T cells was similar to that of anti-CD3 antibody in humans. Ginaldi *et al.* [4] reported that CD4+ T cells exhibit a higher CD3

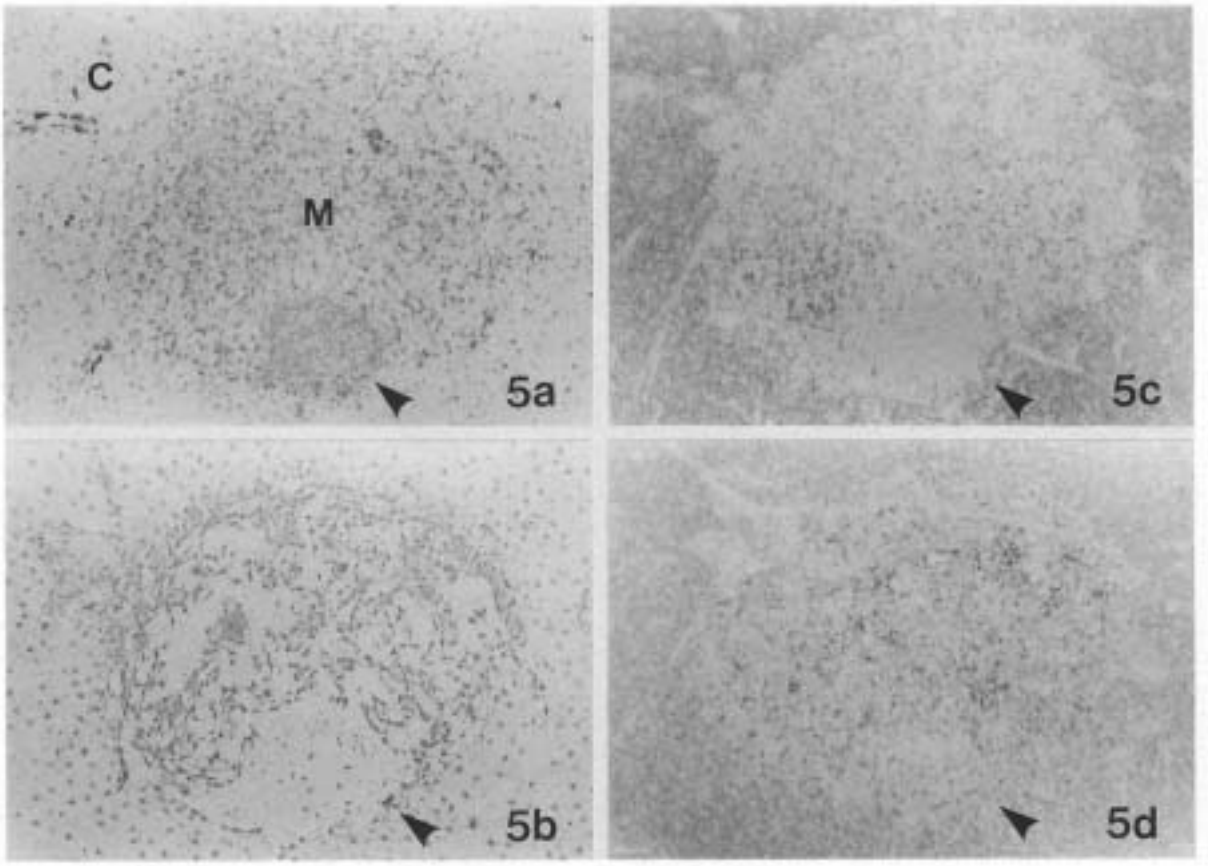

Fig. 5. Four serial sections of the canine thymus stained with antibody 59.4 (5a), anti-cytokeratin (5b), anti-CD8 (5c) and anti-CD4 (5d). An aggregation of 59.4-positive thymocytes (5a, arrowhead) is located on a epithelial reticulum cell-free region (5b, arrowhead). Thymocytes within the aggregation are hardly reactive with anti-CD8 (5c). CD4-positive cells, but not intense in reaction, are scattered within the foci (5d). × 70. C, cortex; M, medulla.

antigen expression compared with $CD8^+$ cells. It is unlikely that the epitope recognized by 59.4 antibody is just the same with CD3 antigen, since, in the present study, the reactivity of $CD8^+$ cells with 59.4 antibody was higher than that of $CD4^+$ cells. At present, we believe that the 59.4 antibody is a new type of monoclonal antibody against lymphocyte, although further analysis including identification of antigen is needed for definite conclusion.

The present immunohistochemistry of the canine thymus using antibody 59.4 demonstrated peculiar aggregations of immunopositive cells in the medulla. The lymphocyte aggregations were characterized by lack of reticulum cells. Therefore, their distribution and shape could be also shown clearly, as an immunonegative image, by immunostaining using antibodies against the epithelial reticulum cells, such as anti-cytokeratin antibodies. No studies have revealed the existence of any reticulum cell-free regions in the medulla of the normal thymus. On the other hand, Bruijntjes *et al.* [1] reported that a reticulum cell-free area exists in the subcapsular region of the rat thymus, and extends from the subcapsular region to the deep cortex, often up to the corticomedullary border. It has also been reported that lymphocyte aggregations, called lymphofollicular hyperplasia or ectopic nodules, are formed in the thymic medulla under pathological conditions which are often associated with autoimmune diseases such as myasthenia gravis [6, 13, 15]. Immunohistochemical examination of lymphofollicular hyperplasia revealed the same cellular composition as in the lymph follicles of the human spleen and lymph node [3, 5]. In the canine thymus, however, no aggregations of 59.4^+ lymphocytes possessed germinal centers. Thus, it was suggested that the lymphocyte aggregations in the canine thymus were not identical to the lymph follicles in some autoimmune diseases.

The functional significance of the reticulum cell-free lymphocyte foci in the canine thymus remains unclear. Epithelial reticulum cells of the thymus are generally believed to play an important role in differentiation and maturation of T cells [11]. Bruijntjes *et al.* [1] have considered that the reticulum cell-free area in the rat thymus may offer a particular intrathymic pathway for T-cell differentiation without contacting and potential selection by cortical stromal elements. In the dog thymus, lymphocytes present in the reticulum cell-free regions were less intensely

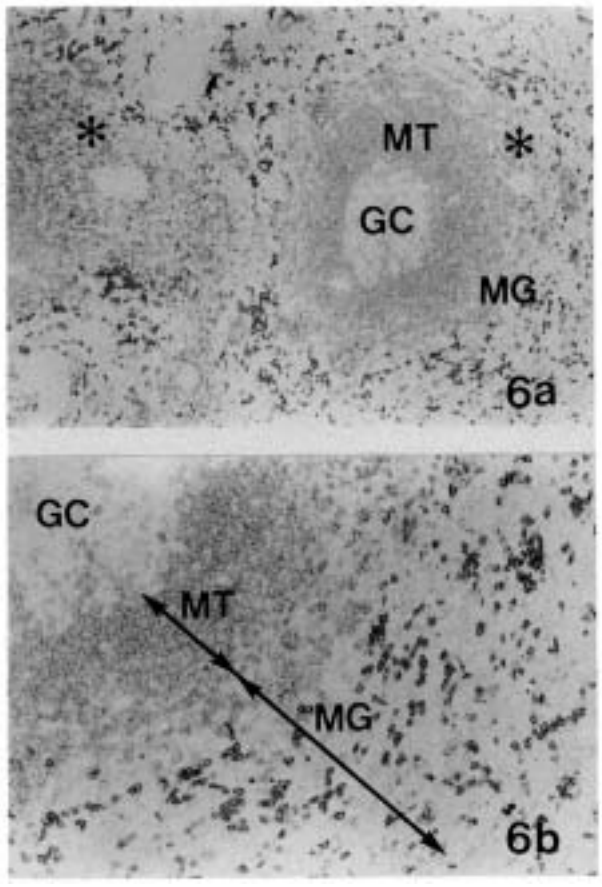

Fig. 6. Immunoperoxidase reaction of canine spleen with antibody 59.4. (6a) Strongly labeled cells are scattered in the outer layer of the marginal zone (MG) and in the red pulp. Small lymphocytes in the mantle zone (MT) and periarterial lymphatic sheath (asterisks) are moderately stained. Germinal center (GC) itself is free from labeling. × 65. (6b) A higher magnification showing the margin of the white pulp. × 130.

reactive with 59.4 antibody than the mature T cells dispersed in the perivascular spaces of the cortex. Therefore, the reticulum cell-free regions in the thymic medulla might serve as a place for production of an unknown T cell subpopulation.

Based on our FCM and immunohistochemical observations, it is concluded that the monoclonal antibody 59.4 detects a surface antigen present on mature T lymphocytes and related to $CD8^+$ cells. This marker of immunocompetent canine lymphocytes can be used to explore the ontogeny of canine T cells.

REFERENCES

1. Bruijntjes, J. P., Kuper, C. F., Robinson, J. E., and Schuurman, H. J. 1993. Epithelium-free area in the thymic cortex of rats. *Dev. Immunol.* 3: 113–122.
2. Cobbold, S. and Metcalfe, S. 1994. Monoclonal antibodies that define canine homologues of human CD antigens: summary of the First International Canine Leukocyte Antigen Workshop (CLAW). *Tissue Antigens* 43: 137–154.
3. Gerdes, J., Stein, H., Mason, P. Y., and Ziegler, A. 1983. Human dendritic reticulum cells of lymphoid follicles: Their antigenic profile and their identification as multinucleated giant cells. *Virchows Arch. B. Cell Pathol.* 42: 161–172.
4. Ginaldi, L., Farahat, N., Matutes, E., De Martinis, M., Morilla, R., and Catovsky, D. 1996. Differential expression of T cell antigens in normal peripheral blood lymphocytes: a quantitative analysis by flow cytometry. *J. Clin. Pathol.* 49: 539–544.
5. Hofmann, W. J., Möller, P., and Otto, H. F. 1987. Thymic hyperplasia. II. Lymphofollicular hyperplasia of the thymus. An immunohistologic study. *Klin. Wochenschr.* 65: 53–60.
6. Hofmann, W. J., Momburg, F., Möller, P., and Otto, H. F. 1988. Intra- and extrathymic B cells in physiologic and pathologic conditions. *Virchows Arch. A, Pathol. Anat. Histopathol.* 412: 431–442.
7. Kato, S. and Schoefl, G. I. 1987. The vasculature of the guinea-pig thymus: topographic studies by light and electron microscopy. *Arch. Histol. Jpn.* 50: 299–314.
8. Köhler, G. and Milstein, C. 1975. Continuous culture of fused cells secreting antibody of predefined specificity. *Nature* (*Lond.*) 256: 495–497.
9. Kydd, J., Antczak, D. F., Allen, W. R., Barbis, D., Butcher, G., Davis, W., Duffus, W. P. H., Edington, N., Grünig, G., Holmes, M. A., Lunn, D. P., McCulloch, J., O'Brien, A., Perryman, L. E., Tavernor, A., Williamson, S., and Zhang, C. 1994. Report of the first international workshop on equine leucocyte antigens, Cambridge, UK, July 1991. *Vet. Immunol. Immunopathol.* 42: 3–60.
10. Lanier, L. L., Allison, J. P., and Phillips, J. H. 1986. Correlation of cell surface antigen expression on human thymocytes by multi-color flow cytometric analysis: implications for differentiation. *J. Immunol.* 137: 2501–2507.
11. Marusic-Galesic, S. and Pavelic, K. 1990. Dynamics of positive and negative selection in the thymus: review and hypothesis. *Immunol. Letters* 24: 149–154.
12. Naessens, J. and Howard, C. J. 1993. Leukocyte antigens of cattle and sheep. Monoclonal antibodies submitted to the second workshop. *Vet. Immunol. Immunopathol.* 39: 5–10.
13. Palestro, G., Tridente, G., Botto Micca, F., Novero, D., Valente, G., and Godio, L. 1983. Immunohistochemical and enzyme histochemical contributions to the problem concerning the role of the thymus in the pathogenesis of myasthenia gravis. *Virchows Arch. B, Cell Pathol. Including Mol. Pathol.* 44: 173–186.
14. Saalmüller, A., Aasted, B., Canals, A., Dominguez, J., Goldman, T., Lunney, J. K., Maurer, S., Pescovitz, M. D., Pospisil, R., Salmon, H., Tlaskalova, H., Valpotic, I., Vizcaino, J. S., Weiland, E., and Zuckermann, F. 1994. Summary of workshop findings for porcine T-lymphocyte antigens. *Vet. Immunol. Immunopathol.* 43: 219–228.
15. Thomas, J. A., Willcox, H.N., and Newsom-Davis, J. 1982. Immunohistological studies of the thymus in myasthenia gravis. Correlation with clinical state and thymocyte culture responses. *J. Neuroimmunol.* 3: 319–335.