Polish Journal of Veterinary Sciences Vol. 13, No. 4 (2010), 661-668

DOI 10.2478/v10181-010-0016-1

Original article

Practical aspects of immunocytochemistry in canine lymphomas

R. Sapierzyński

Department of Pathology and Veterinary Diagnostic, Faculty of Veterinary Medicine, Warsaw University of Life Sciences (SGGW), Nowoursynowska 159c, 02-766 Warsaw, Poland

Abstract

The aim of this study was to evaluate the utility of immunocytochemistry in a standard veterinary practice and to determine the immunophenotype of tumor cells in cases of multicentric lymphoma in dogs by immunocytochemical analysis of fine-needle biopsy specimens. The study was performed on cytological samples collected from 54 dogs, in which multicentric lymphoma was recognised based on clinical data, cytology or cytology and histology, and follow-up information. Diagnosis of lymphoma was established according to the updated Kiel classification. Immunocytochemical assays were conducted using commercially available antibodies to the pan T-lymphocyte marker CD3 and B cell antigen receptor complex CD79 alpha. Among all animals examined B cell lymphoma was recognized in 42/54 (77.8%) of cases, while in the remaining 12/54 (22.2%) of dogs T cell lymphoma was recognized. In 11 animals with lymphoma recognized cytologically, in which an entire lymph node was obtained for histology, the results of routine cytology and immunocytochemistry fully corresponded with findings revealed by histology and immunohistochemistry. Immunocytochemistry can be successfully conducted in smears stored at room temperature for 24 hours without changes of staining results. It can be stated that application of standard cytopathological assessment in connection with immunocytochemistry of lymph nodes samples collected from dogs with lymphoma is a method of choice for establishing final diagnosis, and avoids the need for reexamination or collection of tissue samples for histopathology and immunohistochemistry during surgical procedures in ambiguous cases.

Key words: canine lymphoma; cytology; immunocytochemistry; immunophenotype

Introduction

Lymphomas are one of the most common malignancies recognised in dogs and major clinical manifestation in these tumours is single, regional or systemic lymphadenomegaly (Foulner-Fleury et al. 2002, Pastor et al. 2008, Sapierzyński and Micuń 2009). The first step in the diagnostic procedure in these cases should be a cytological examination of an enlarged lymph node (Teske and van Heerde 1996, Foulner-Fleury et al. 1997, Das 1999, Foulner-Fleury et al. 2002, Sozmen et al. 2005, Guija de Arespacochoga et al. 2007, Sapierzyński and Micuń 2009). In some cases establishing of final diagnosis of lymphoma requires histopathological examination of the entire or sample of lymph node collected during surgery. However, this diagnostic procedure can be impossible or risky in some animals because of their poor general state or lack of owner's consent. Thus, there is the need to discover new, less invasive methods of examination, which contribute more to the diagnostic process and will allow other more invasive procedures to be avoided.

Correspondence to: R. Sapierzyński, e-mail: rafal_sapierzynski@sggw.pl, tel.: +48225936153

Determining of the immunophenotype of the lymphoma cells is possible by using specific mono- and polyclonal antibodies for surface cluster of differentiation (CD) antigens. CD markers and markers of the stage of maturity are very often used in the human lymphoma diagnostic process. Additionally, immunocytochemistry allows the course of disease to be monitored and, often, most suitable treatment to be chosen. Selected sets of monoclonal antibodies can detect antigens that are specific markers of cells lines and allow them to be differentiated and the level of their maturity and immunophenotype to be determined. Immunocytochemical staining (IC) of samples collected by fine needle biopsy enables fast, relatively cheap and direct definition of human and canine lymphoma immunophenotype (Vail et al. 1997, Caniatti et al. 1999, Sozmen et al. 2005).

The aim of this study was to evaluate the utility of immunocytochemistry in routine veterinary practice and determine the immunophenotype of lymphoma cells in quite numerous cases of multicentric lymphoma in dogs.

Materials and Methods

The study was performed on cytological samples collected from 54 dogs presented to the Small Animal Clinic, Faculty of Veterinary Medicine, Warsaw University of Life Sciences, in which multicentric lymphoma was recognised based on clinical data, cytology or cytology and histology, and follow-up information. None of the dogs had received chemotherapy previously. In some of the examined animals antibiotic or anti-inflammatory therapy was conducted; however, in any case, reduction of lymph node size was observed.

Samples for cytological examination were obtained by fine-needle aspiration or fine-needle nonaspiration biopsy of enlarged lymph nodes (at least two enlarged lymph nodes were examined and at least four samples from each examined lymph node were taken). For routine examination at least 3 smears of aspirates were dried, fixed in 70% methanol, stained with Giemsa stain and examined by light microscope. Diagnosis of lymphoma was established according to the updated Kiel classification, assuming that at least 80% of the cells present on slides are blastic cells (excluding lymphocytic lymphomas). For immunocytochemical assays smears from each dog were dried, fixed in acetone at 4°C for 5-10 minutes, and stained immediately or stored at -20°C. Immunocytochemical stains were conducted according to Caniatti et al. (1996) using commercially available antibodies (Dako® Denmark) to the pan T-lymphocyte marker CD3 (Polyclonal Rabbit Anti-Human) and B cell antigen receptor complex CD79 alpha (Monoclonal Mouse Anti-Human). Four smears from the same lymph nodes were stained using both antibodies.

Expression intensity of examined CD antigens in cytological preparation was determined on the basis of microscopic light evaluation as follows: (+) when at least 80% of cells showed strong cytoplasmic reaction, (+-) when less than 80% of cells showed strong cytoplasmic reaction, (-) when less than 10% of cells showed strong cytoplasmic reaction, (-/+) when 10-15% of cells showed strong cytoplasmic reaction and (-/++) when 15-20% of cells showed strong cytoplasmic reaction.

In 11 of these cases, the results of immunocytochemical assays (IC) were compared with immunohistochemical (IH) analysis of the entire lymph node obtained during surgery. Before surgical removal, routine fine-needle biopsy was performed on one of the affected lymph nodes, and the lymph node was then obtained surgically, fixed in 10% neutral buffered formalin, embedded in paraffin wax, cut in sections (3 µm) and stained with hematoxylin and eosin. For immunohistochemical staining, tissue samples were processed in the same way. The expression of the CD3 and CD79 alpha antigens was stained using the same primary antibodies as mentioned above. Briefly, 3-µm-thick sections on 2% silane coated slides were deparaffinized in xylenes and hydrated through alcohol gradients. Antigen unmasking was performed by microwave heating at 600W for 15 min. in 10 mM sodium citrate buffer, pH 6.0 and Tris/EDTA buffer. Sections were allowed to cool in the buffer at room temperature for 20 min and were rinsed in deionized H_2O twice, 2 min each. The endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 5 min. Sections were incubated with antibodies for 1 h at room temperature in a humid chamber, and after extensive washing in Tris-buffered saline (TBS) (0.1 M Tris base, 0.9% NaCl, pH 7.4) were further incubated with a biotinylated secondary antibody. The following procedures were then carried out according to standard protocols with EnVision™ System (Dako®, Denmark). The reactions were developed with 3-3'diaminobenzidine (Dako®, Denmark), under microscopic control. Sections were counterstained with Mayer's hematoxylin, dehydrated, cleared in xylene and mounted. Positive and negative immunohistochemical controls were performed. Tissue sections of formalin-fixed, paraffin-embedded normal canine lymph nodes were treated as positive controls in every assay. Corresponding negative control sections were prepared by replacing only the primary antibody with TBS.

To evaluate the possible influence of smear storage condition on IC results, in 10 cases additional smears were stored for 24 hours after collection at room temperature, beside staining of fresh and frozen

smears cold acetone fixed, and just after this time smears were fixed according to described procedure on immunocytochemical fixing and staining. The results of staining of these smears were compared with the same patient's smears which were fixed immediately after collection and frozen.

Results

Lymphoma was recognized in 54 dogs that were examined at the Small Animal Clinic because of mild to severe regional or general lymphadenomegaly. In some animals clinical symptoms of disease were observed and in some animals enlarged lymph nodes were the only clinical manifestation. Among 54 dogs examined B cell lymphoma (Fig. 1) was recognized in 42/54 (77.8%) of cases, in the remaining 12/54 (22.2%) of animals T cell lymphoma (Fig. 2) was recognized. No non-B and non-T cell lymphoma was recognized. In all 11 animals, with lymphoma recognized on the basis of cytology and immunocytochemistry, from which a lymph node was obtained during surgery, histopathology and immunohistochemistry confirmed diagnosis and the microscopic picture of histologic sections corresponded with those observed during the cytological tests. In all cases the immunophenotype of tumor determined by IH corresponded with that defined earlier by IC. However, in some cases some differences between the number of cells stained in some areas of affected lymph nodes were observed (Table 1, Figs. 3, 4). IC results were similar in all smears prepared from the same lymph nodes fixed directly after collection, compared with these stored at room temperature for 24 hours and fixed after this time.

B cell lymphomas (42 cases) were recognized in dogs between 3 and 14 years old (average age 8.2), 26 males (62%) and 16 females (38%), various breeds (5 schnauzer, 3 golden retriever, 3 Bernese mountain dogs, 3 German shepherds, 2 briards, and 1 each of 13 other breeds) and 13 mongrels. Among all recognized B cell tumors 6 (14.3%) were low-grade lymphomas (3 small cell lymphomas, 3 macronucleated medium-sized cell lymphomas) and 36 (85.7%) were high-grade lymphomas (34 centroblastic monomorphic and polymorphic type lymphomas, and 2 small blastic lymphomas). Moderate or more often strong CD 79 alpha antigen expression was established as (+) in all of these B cell lymphomas (Fig. 1), additionally in 28 cases (66.7%) expression of CD3 antigen was established as (-), in 10 cases (23.8%) as (-/+) and in 4 cases (9.5%) as (-/++).

T cell lymphomas (12 cases) were recognised in animals aged between 1.5 and 11 years old (average age 5.4), 7 males and 5 females, of various breeds (4 boxers, and 1 each of 6 other breeds) and two mongrels. Among them 3 (25%) were of low-grade lymphomas (all small cell lymphomas) and 9 (75%) were high-grade lymphomas (4 pleomorphic mixed small and large cell lymphomas, 2 lymphoblastic lymphomas, 2 unclassifiable plasmacytoid lymphomas, and 1 unclassifiable small blastic lymphoma). Strong CD 3 antigen expression was established as (+) in all of these T cell lymphomas (Fig. 2). Additionally in 11 cases (91.6%) expression of CD 79 alpha antigen was established as (-), in 1 case (8.4%) as (-/+), there were no lymphomas with CD 79 alpha expression established as (-/++).

Discussion

Application of the immunostaining methods in diagnosis of canine cancers, especially in the case of lymphoma, is widely described. However, in most

Table 1. Comparison of intensity of lymphocyte expression with immunophenotype different to tumor cell immunophenotype in cytological and histological preparation from canine lymphomas. (-) less than 10% of cells showed reaction; (-/+) 10-15% cells showed reaction: (-/++) 15-20% cells showed reaction.

Case	Immunocytochemistry	Immunohistochemistry
B cell lymphomas	CD3 antigen expression	CD3 antigen expression
No. 1	(-)	focally (-/+) small mature lymphocytes
No. 2	(-)	(-)
No. 3	(-)	(-)
No. 4	(-/+) small mature lymphocytes	(-+) small mature lymphocytes
No. 5	(-)	focally (-/+) small mature lymphocytes
No. 6	(-/+) small mature lymphocytes	(-/+) small mature lymphocytes
No. 7	(-/+) small mature lymphocytes	(-/+) small mature lymphocytes
No. 8	(-)	(-)
T cell lymphomas	CD79 alpha expression	CD79 alpha expression
No. 9	(-)	(-)
No. 10	(-)	focally (-/+) small mature lymphocytes
No. 11	(-/+) small mature lymphocytes	focally (-/+) small mature lymphocytes

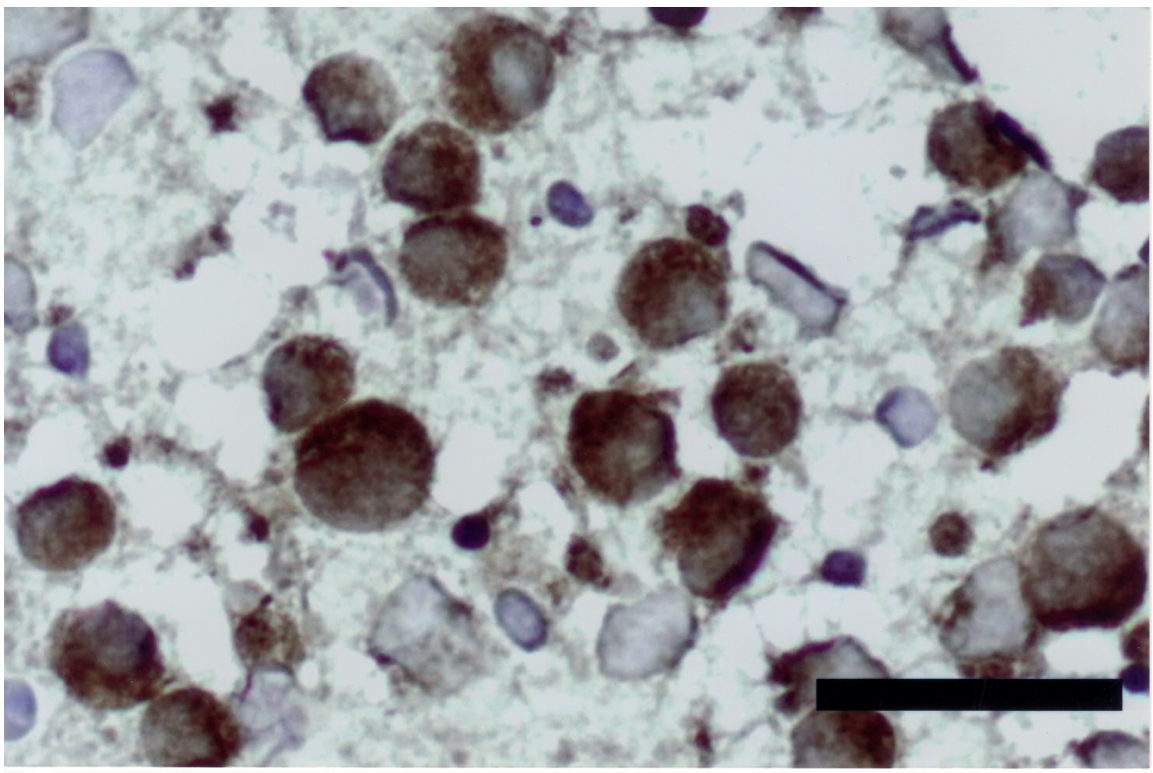

Fig. 1. Lymph node aspirate of dog with centroblastic polymorphic lymphoma. Fine-needle aspiration biopsy. Most of the cells observed of this B-cell lymphoma show strong CD79 alpha cytoplasmic expression. Immunoperoxidase, Mayer's hematoxylin counterstain. Bar = 50 µm.

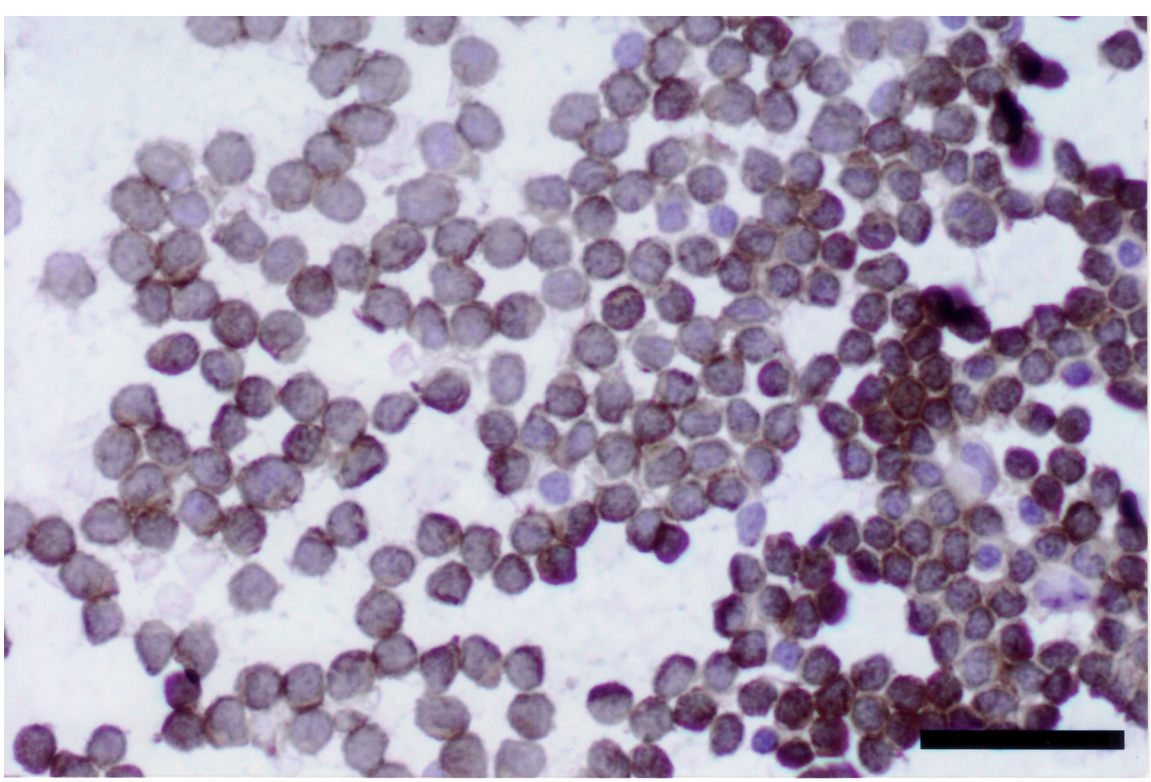

Fig. 2. Lymph node aspirate of dog with small cell lymphoma. Fine-needle aspiration biopsy. Virtually all observed cells of this T-cell lymphoma show strong CD3 cytoplasmic expression. Immunoperoxidase, Mayer's hematoxylin counterstain. Bar = 50 µm.

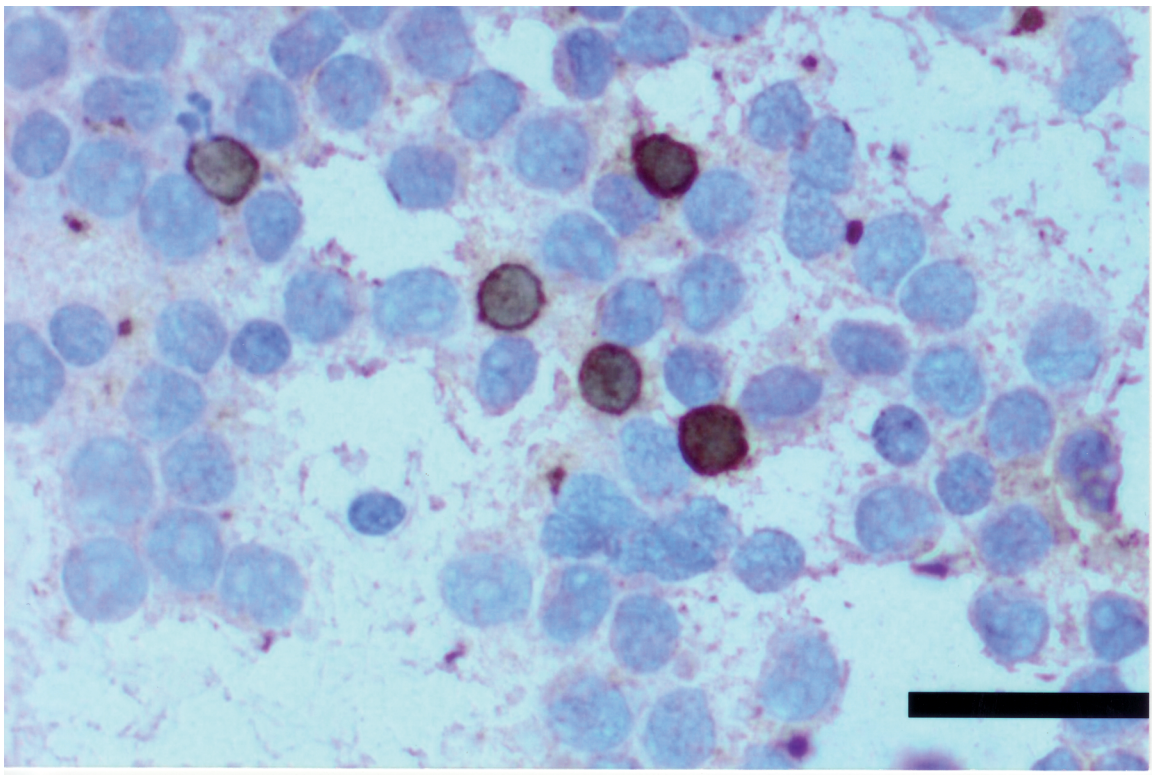

Fig. 3. Lymph node aspirate of dog with centroblastic polymorphic lymphoma (CD79 alfa immunophenotype). Fine-needle aspiration biopsy. Only a small number of cells observed show strong CD3 cytoplasmic expression. Immunoperoxidase, Mayer's hematoxylin counterstain. Bar = 50 μm.

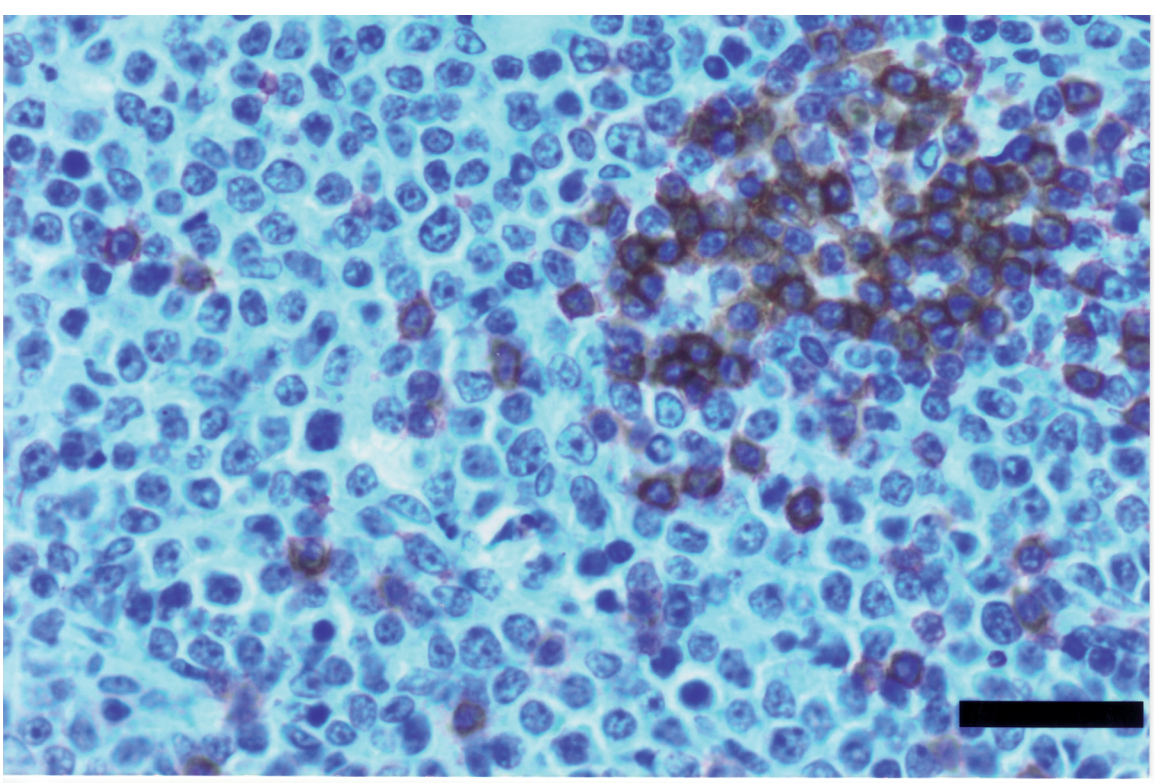

Fig. 4. Canine centroblastic polymorphic lymphoma (CD79 alfa immunophenotype; the same case as in Fig. 3). Tissue section. Note small group of normal T lymphocytes (upper right) in area of tumor proliferation (left and at bottom) only a few cells showing strong CD 3 cytoplasmic expression are present. Immunoperoxidase, Mayer's hematoxylin counterstain. Bar = 50 μm.

cases the immunophenotype of tumor cells was established based on immunohistochemical stains of tissue sections of lymph nodes obtained during surgery or by using flow cytometry and cytospin preparations (Gibson et al. 2004, Sueiro et al. 2004, Jubala et al. 2005, Sozmen et al. 2005, Lana et al. 2006, Dzimira 2007, Guija de Arespacochaga et al. 2007, Williams et al. 2008). The possibility of using smears of cytologic samples obtained by fine-needle aspiration biopsy to assess the immunophenotype of lymphoid cells was shown in dogs with recognized lymphoma, reactive growth and in patients without changes observed in lymph nodes (Fisher et al. 1995, Vail et al. 1997). Various antibodies revealing antigen expression typical for some types and subtypes of cells, including immunological system cells, were used in these examinations.

Determining of antigen CD79alpha and/or antigen CD21 expression, as well as immunoglobulin expression when there is an absence of CD3 antigen expression, allows an examined lymphoid tumor to be qualified as B cell lymphoma. CD3 antigen presence, with simultaneous expression of one or more antigens such as CD4, CD5, CD8, with simultaneous lack of CD79a antigen expression, allows T cell lymphoma to be diagnosed. In a recently published immunohistochemical analysis, B cell lymphoma or T cell lymphoma were recognized if more than 60% of cells within a given growth showed expression CD79 alpha antigen or CD3 antigen, respectively (Guija de Arespacochaga et al. 2007). In the present immunocytochemical study these criteria were more rigorous and it was necessary to state that at least 80% of cells showed a positive reaction to qualify the lymphoma to a direct immunophenotype; it appears that in all examined cases this high percentage of positively reacting cells was observed. In most cases the percentage of cells with the same phenotype was even higher and reached over 90% of all observed cells in examined smears, especially in T cell lymphomas.

It is possible that collected cytological samples and tumor cell immunophenotype determined by immunocytochemistry may not be representative for the entire affected lymph node, especially when only one smear was stained. Therefore, in the present study IC results were compared with immunohistochemical stain results of the entire lymph node obtained during surgery. The results of IC and IH stains conducted in the same patients showed no important differences between intensity of antigen expression that could influence the diagnosis of recognized lymphoma immunophenotype. For example: in some cases of B cell lymphomas, where IC tests showed no cell with CD3 antigen expression (-) or expression which was established as (-/+), IH assays showed presence of larger clusters of these cells (CD3 +) scattered between or present within areas of the lymphoma parenchyma. However, only a minimal number of CD3+ cells was shown in areas of cancer growth, similarly to those observed in cytological preparation. Fisher and al. (1995) found full correlation between IC staining results of samples obtained by fine-neeedle aspiration biopsy, and IH staining results of small samples obtained by needle biopsy. Similarly, 100% concordance was obtained in a study including 51 dogs with lymphoma in which both IC and IH with use of anti-CD3 antibody were performed (Vail et al. 1997).

Additionally, results of immunocytochemical staining (as the other additional tests results) should be considered in close relation to results of examination of smears stained with basic methods (Giemsa stain in the present study). Moreover, immunocytochemistry provides not only the possibility to assess the presence or absence of reaction, but also determines the morphology of cells in which antigen expression is observed or not. In smears stained with IC it is possible to determine which cells are small mature lymphocytes and are a part of the normal cells of the lymph node population, and which should be recognized as lymphoma cells. The present study revealed that in smears which were stained using a different antibody than the immunophenotype of the examined tumor (CD3 staining in smears of B cell lymphoma and vice versa) the majority of the stained cells were small and well differentiated cells that were considered to be normal, nonneoplastic cells of the affected lymph node.

To make the IC assays more objective to assess, lymphoma immunophenotype tests using both antibodies on the various parts of the one smear may be performed and the test can be repeated in another smear. Thus, two different smears are assessed for the presence of both CD3 and CD 79 alpha antigens. As the present study revealed, use of this technique with good quality smears gives repetitive results when compared with IH and IC results.

The application of updated Kiel classification for assessment of cytologic smears stained using routine methods such as Giemsa staining allows not only the lymphoma subtype to be determined but also indicates its immunophenotype (for example: MMC and centroblastic lymphomas – B cell immunophenotype; clear cell lymphoma – T cell immunophenotype). In some cases (immunoblastic lymphoma, small cell lymphoma, anaplastic lymphoma) determining of tumor cell immunophentype based on Giemsa stain only is very difficult or impossible. In the present study in most cases when determining of lymphoma subtype caused some difficulties, IC results indicated T immunophenotype. In most of these cases routine recognition was blastic lymphomas or mixed small and large cell lymphomas, rarely small cell lymphomas. Unfortunately, no immunoblastic lymphoma was recognized in the present study, in which IC seems to be especial-

ly helpful in immunophenotype establishment. It should, however, be stated that IC should be helpful regardless of morphologic subtype of lymphoma.

Immunocytochemical staining of smears obtained by fine needle biopsy has many advantages. It is easy to perform, relatively cheap and the result can be obtained on the same day that samples were collected. Additionally, as the present study showed, obtained smears do not need to be immediately fixed and stored in conditions that are impossible to meet in many veterinary practices. Samples can be collected and then sent to the laboratory within 24 hours, without the need for special transportation; this is especially attractive for routine veterinary practice. In 10 examined cases no difference in results of IC was observed in comparison to samples fixed according to the Cianiatti et al. (1996) method – immediately after collection. Impossibility of smear quality initial assessment for smears chosen to be stained by IC (which can make analysis after staining impossible because of poor quality of samples), is the main disadvantage of this method. The gross description of the slide does not always correlate with the microscopic quality of the smear. Furthermore, lymphoma cells are relatively sensitive to damage and quite often stained fragments of cytoplasm among undamaged cells can be observed on the smears, giving a "dirty background" image. This can be avoided by staining cytospin preparations obtained by fine-needle biopsy (Cianiatti et al. 1996). On the other hand this "dirty background" can be considered as a positive reaction indicator, since the "dirty background" was not observed in slides of T cell lymphoma stained by antibody CD 79 alpha and, vice versa, there was no "dirty background" in B cell lymphoma slides stained with CD 3 antibody.

The majority of studies performed on canine lymphomas determined that B cell tumors are more common (51-64%) then T cell lymphomas (26-38% lymphomas in dogs) (Jagielski et al. 2002, Sueiro et al. 2004, Sokołowska 2005, Dzimira 2007, Guija de Arespacochaga et al. 2007). The results of the present study confirmed this observation, and also indicate that B cell lymphomas are more common – more then 77% of cases. Observed differences may be the result of the small number of dogs examined, or the fact that most of the dogs included in the study could be predisposed to B cell lymphoma development. Recent epidemiological research conducted on dogs from France indicated a clear predisposition of boxer dogs for T cell lymphoma development and German shepherd and rottweiler for B cell lymphomas (Pastor et al. 2009). Although the present study does not include the epidemiological aspect, it at least partially supports the French authors observation, since in all boxers examined only T cell lymphomas were recognized (4 cases – 33.3% of all T cell lymphomas) and in all German shepherds (3 cases – 7.1% of all B cell lymphomas) only B cell lymphomas were recognized.

In summary, it can be stated that the application of standard cytopathological assessment and immunocytochemistry of lymph node samples collected from dogs with multifocal lymphoma can be recommended as useful for establishing final diagnosis, especially in cases of ambiguous basic staining results. The connection of basic staining with IC can be considered as a method of choice that avoids the need for reexamination or collection of tissue samples for histopathology and immunohistochemistry during surgical procedures.

References

Caniatti M, Roccabianca P, Scanziani E, Paltrinieri S, Moore PF (**1996**) Canine lymphoma: immunocytochemical analysis of fine-needle aspiration biopsy. Vet Pathol 33: 204-212.

Das DK (**1999**) Value and limitations of fine-needle aspiration cytology in diagnosis and classification of lymphomas. A review. Diagn Cytopathol 21: 240-249.

Dzimira S (**2007**) Immunocytochemical and cytomorphometric diagnostics of malignant lymphomas in dogs. Bull Vet Inst Pulawy 51: 71-78.

Fisher DJ, Naydan D, Werner LL, Moore PF (**1995**) Immunophenotyping lymphomas in dogs: a comparison of results from fine needle aspirate and needle biopsy samples. Vet Clin Pathol 24: 118-123.

Fournel-Fleury C, Magnol JP, Bricaire P, Marchal T, Chabanne L, Delverdier A, Bryon PA, Felman P (**1997**) Cytohistological and immunological classification of canine malignant lymphomas: comparison with human non-Hodgkin's lymphomas. J Comp Pathol 117: 35-59.

Fournel-Fleury C, Ponce F, Felman P, Blavier A, Bonnefont C, Chabanne L, Marchal T, Cadore JL, Goy-Thollot I, Ledieu D, Ghernati I, Magnol JP (**2002**) Canine T-cell lymphomas: a morphological, immunological, and clinical study of 46 new cases. Vet Pathol 39: 92-109.

Gibson D, Aubert I, Woods JP, Abrams-Ogg A, Kruth S, Wood RD, Bienzle D (**2004**) Flow cytometric immunophenotype of canine lymph node aspirates. J Vet Intern Med 18: 710-717.

Guija de Arespacochaga A, Schwendenwein I, Weissenbock H (**2007**) Retrospective study of 82 cases canine lymphoma in Austria based on the Working Formulation and immunophenotyping. J Comp Pathol 136: 186-192.

Jagielski D, Lechowski R, Hoffman-Jagielska M, Winiarczyk S (**2002**) A retrospective study of the incidence and prognostic factors of multicentric lymphoma in dogs (**1998-2000**). J Vet Med A 49: 419-424.

Jubala CM, Wojcieszyn JW, Valli VE, Getzy DM, Fosmire SP, Coffey D, Bellgrau D, Modiano JF (**2005**) CD20 expression in normal canine B cells and in canine non-Hodgkin lymphoma. Vet Pathol 42: 468-476.

Lana S, Plaza S, Hampe K, Burnett R, Avery AC (**2006**) Diagnosis of mediastinal masses in dogs by flow cytometry. J Vet Intern Med 20: 1161-1165.

Pastor M, Chalvet-Monfray K, Marchal T, Keck G, Magnol JP, Fournel-Fleury C, Ponce F (**2009**) Genetic and environmental risk indicators in canine non-Hodgkin's lymphomas: breed association and geographic distribution of 608 cases diagnosed throughout France over 1 year. J Vet Intern Med 23: 301-310.

Ponce F, Magnol JP, Ledieu D, Marchal T, Turinelli V, Chalvet-Monfray K, Fournel-Fleury C (**2004**) Prognostic significance of morphological subtypes in canine malignant lymphomas during chemotherapy. Vet J 167: 158--166.

Sapierzyński R, Micuń J (**2009**) Lymphadenomegaly in dogs – cytological study. Pol J Vet Sci 12: 263-268.

Sokolowska J, Cywińska A, Malicka E (**2005**) p53 expression in canine lymphoma. J Vet Med A 52: 172-175.

Sozmen M, Tasca S, Carli E, De Lorenzi D, Furlanello T, Caldin M (**2005**) Use of fine needle aspirates and flow cytometry for the diagnosis, classification, and immunophenotyping of canine lymphomas. J Vet Diagn Invest 17: 323-330.

Sueiro FA, Alessi AC, Vassallo J (**2004**) Canine lymphomas: a morphological and immunohistochemical study of 55 cases, with observations on p53 immunoexpression. J Comp Pathol 131: 207-213.

Teske E, van Heerde P (**1996**) Diagnostic value and reproducibility of fine-needle aspiration cytology in canine malignant lymphoma. Vet Q 18: 112-115.

Vail DM, Kravis LD, Kisseberth WC, Ogilvie GK, Volk LM (**1997**) Application of rapid CD3 immunophenotype analysis and argyrophilic nucleolar organizer region (Ag-NOR) frequency to fine needle aspirate specimens from dogs with lymphoma. Vet Clin Pathol 26: 66-69.

Williams MJ, Avery AC, Lana SE, Hillers KR, Bachand AM, Avery PR (**2008**) Canine lymphoproliferative disease characterized by lymphocytosis: immunophenoptypic markers of prognosis. J Vet Intern Med 22: 596-601.