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MORPHOLOGICAL, HISTOPATHOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY OF CANINE MALIGNANT LYMPHOMA

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Lymphoid tumours comprise one of the most common groups of tumours in dogs and there was considerable confusion with regard to their nomenclature and classification. A new classification of lymphoid neoplasm was proposed by the International Lymphoma Study Group and is accepted as the Revised European American Lymphoma Classification-R.E.A.L. Classification. These tumours are divided into three major categories: B cell neoplasm, T cell and natural killer neoplasm and Hodgkin's disease.

Canine malignant lymphoma was detected 11 cases out of 119 dogs of different age, sex and breed, necropsied in the last two years at the Department of Pathology, Faculty of Veterinary Medicine, Belgrade. According to the anatomical classification, three different forms of lymphoma were seen: the most frequent form was multicentric (8 of 11 cases), followed by alimentary form (2 of 11 dogs) whereas cutaneous lymphoma occurred in 1 dog. Histologically, three different forms of lymphoma were found: poorly differentiated (in 6 cases), intermediate (in 4 cases) and well differentiated (in 1 case).

By immunohistochemical demonstration of CD79, CD3 and MAC-387, we showed that B-cell lymphomas were predominant (8 of 11 cases), while T cell lymphomas were demonstrated in 3 cases.

Key words: dog, immunohistochemistry, lymphoma.

INTRODUCTION

Haematopoietic tumours, in particular those originating from the lymphatic tissues, are among the most frequently occurring neoplasm in the dog. Canine lymphoma has often been proposed as an animal model of its human counterpart, the non-Hodgkins lymphomas (NHL), also because of the fact that this species shares the environment of man (Teske, 1994). Lymphohaemopoiesis proceeds postnatally from pluripotent stem cells in the bone marrow. Neoplasias that are composed of cells from several cell lineage should result from the transformation of pluri-or multipotent stem-cells, whereas neoplasias consisting of a single cell lineage might result from the transformation of a subsequent stem cell that is only unipotent (Breuer and Hermanns, 1998).

The lymphohaemopoietic neoplasias consist of two main groups, myeloid and lymphatic neoplasias. A new classification of lymphoid neoplasm has been proposed by the International Lymphoma Study Group and accepted as the Revised European American Lymphoma Classification - R.E.A.L. Classification (Harris *et al.* 1994).

The incidence rate of canine NHL is not the same for all breeds. Increased relative risks have been reported for boxers, Scottish terriers, Basset hounds, Airedale terriers, and bulldogs, whereas the breeds with lower relative risk include dachshunds and Pomeranians (Preister, 1967; 1984). The breed predisposition might suggest that NHL has a genetic basis. Lymphomas affect dogs of all ages, but predominantly those in middle-age. The average age of dogs with NHL has been reported to vary from 6,3-7,7 years (Madewell, 1986; Haga *et al.*, 1988). Although a female predominance was observed by some authors, no sex predilection was revealed in most studies. In humans the incidence of NHL is slightly but consistently higher in men than in women (Bernard, 1988). Canine malignant lymphoma (CML) has many similarities to NHL in humans and it offers an excellent model for new chemotherapeutical drugs. Because of increasing possibilities for proper diagnosis and treatment, these tumours have become of considerable interest to the veterinary profession.

MATERIAL AND METHODS

Eleven dogs of different age, breed and sex, with malignant lymphoma, were necropsied at the Department of Pathology, Faculty of Veterinary Medicine, Belgrade. Tissue samples from lymph nodes, spleen, liver, kidneys, intestine and lungs were fixed for 24 - 48h in 10% neutral formaline and embedded in paraffin. The tissue sections, of thickness approximately 5 μm, were stained with haematoxylin and eosin and by the Giemsa method for histological analyses. The tissue specimens from these eleven dogs with lymphoma diagnosed by routine histological methods, were also used for immunohistochemical examinations. Tissues from three negative dogs served as the negative control.

Immunohistochemistry. The peroxidase-anti peroxidase method (PAP) was applied to the formaline fixed material. Briefly, 5 μm thick sections were deparaffinized in xylene and rehydrated through graded alcohol. Endogenous peroxidase was blocked by incubation with 0,3% H_2O_2 in methanol at room temperature for 30 minutes. Sections were then washed in TRIS-buffered saline (TBS, 0,1M TRIS-HCl, 0,9%NaCl, pH=7,8) and treated with protease (P8038, Sigma), freshly diluted in PBS (pH=7,2) at 37 ^{0}C for 5 minutes and washed again in TBS. After that the sections were incubated with 10% rat serum in TBS, at room temperature, for 10 minutes, followed by 12-16h incubation at 4 ^{0}C, with primary antibodies. The monoclonal mouse - anti human CD79 (Dako, M7051) for the B cell lineage, and mouse - anti human MAC-387 (Dako M0747) for macrophages, monocytes and neutrophil granulocytes, were each applied separately. Monoclonal mouse antibodies (Kontr.T1-Inst.Vet.Path, Giessen) were used as the negative control.

The direct peroxidase (DP) method was used to detect CD3 antigens on the T lymphocytes in different tissue sections. Specimens underwent the process of proteolytic demasking of antigens by trypsinization (for 35 minutes at 37 ^{0}C), using commercial lyophilised trypsin from cattle pancreas (Dako, S2012). Endogenous

peroxidase (EP) activity was quenched in 3% H_2O_2 in distilled water for 5 minutes. In DP method rabbit-anti human T cells horseradish peroxidase coupled commercial antibodies (Dako epos CD3/HRP, U0026) were used. The commercial Dako EPOS negative control (Dako, U0951) was used as the negative control for CD3.

For the monoclonal antibodies, sections were incubated with rat anti-mouse Ig for 30 minutes at room temperature, and after that with mouse PAP for 30 minutes at room temperature as well. Between each incubation step slides were washed with TBS. All sections were incubated for 10 minutes with 0,05% 3,3-diaminobenzidine tetrachydrochloride (DAB,Serva) in 0,1 M buffered imidazole HCl, (pH=7,1), and counterstained with Papanicolaou haematoxylin (Merck).

RESULTS

The incidence of canine malignant lymphoma (CML) was 11 cases out of 119 dogs of different age, sex and breed, necropsied in the last two years (Table 1).

Table 1. Forms of CML in dogs of different ages, breeds and sexes.

No.	Breed	Sex	Age yrs.	Anat. form	Histological form	Immuno-phenotype
1.	Boxer	F	6	MC	well (LC) differentiated	T-cell
2.	English Cocker Spaniel	M	7	MC	poorly (LB) differentiated	B-cell
3.	Giant Schnauzer	F	8	C	intermediate (PLC)	T-cell
4.	Rottweiler	M	8	MC	poorly (LB) differentiated	B-cell
5.	Rottweiler	M	3	A	poorly (LB) differentiated	B-cell
6.	Staffordshire Terrier	M	5	MC	intermediate (PLC)	B-cell
7.	Doberman	F	5	MC	poorly (LB) differentiated	B-cell
8.	English Cocker Spaniel	M	6	MC	poorly (LB) differentiated	B-cell
9.	Mixed breed	M	5	MC	intermediate (PLC)	B-cell
10.	Great Dane	M	4	MC	intermediate (PLC)	T-cell
11.	Mixed breed	M	6	A	poorly (LB) differentiated	B-cell

MC - multicentric, C - cutaneous, A - alimentary

According to the anatomical classification, three different forms of lymphoma were seen: the most frequent form was multicentric (8 of 11 cases) followed by the alimentary form (in 2 cases) and cutaneous lymphoma (*Mycosis fungoides*) in 1 dog. Gross morphological changes included enlarged neoplastic lymph nodes which varied in diameter from 2-6 cm. They were usually non adherent, although mesenteric nodes (in the alimentary form) were fused. The demarcation between cortex and medulla was lost and the tissue was smooth and glistening on the cut surface. The colour was grey, cream or light tan. In a few cases the spleen had multiple nodular masses resembling enlarged follicles and in some cases showed diffuse involvement with marked enlargement. The affected livers were uniformly enlarged with disseminated small pale foci and in one case it contained multiple, large, pale tumour nodules. The kidneys in one case showed multiple white to cream-coloured nodules in the cortex and in the other cases they were diffusely enlarged and pale (Figure 1).

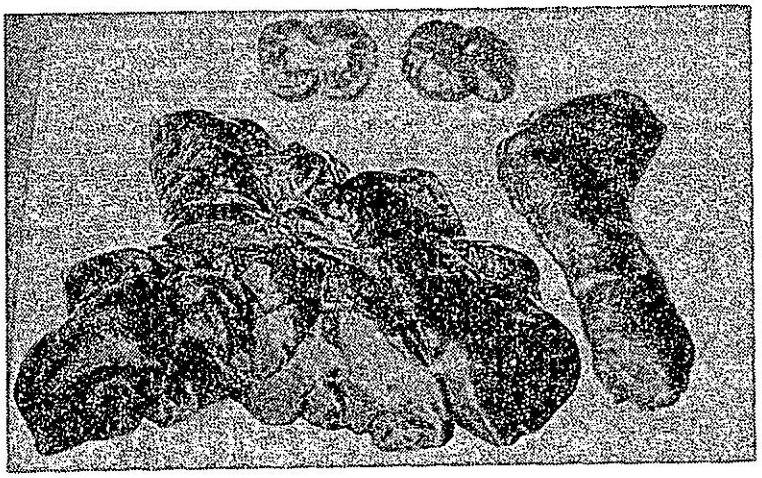

Figure 1. Canine malignant lymphoma. The spleen showed diffuse involvement with marked enlargement and a few nodular masses. The liver was uniformly enlarged with disseminated pale foci. The kidneys were diffusely enlarged and pale. (Gross morphology).

In the intestine of two dogs the neoplasm had diffusely invaded throughout the wall.

Histologically, three different forms of lymphoma were seen: poorly differentiated (in 6 cases), intermediate (in 4 cases) and well differentiated (in 1 case). The architecture of the lymph nodes was usually completely effaced, by neoplastic cells. Capsular invasion was common. Nodular aggregations of tumour cells were detected in the cortex in some cases. Two patterns of histological involvement were seen in the spleen. One was focal localisation, particularly involving the germinal centre region of the lymphatic follicle and the other

involvement was in the red pulp with a diffuse pattern. In the liver there was accumulation of neoplastic cells in the portal triads and extramedullary haematopoiesis was present in one dog (Figure 2).

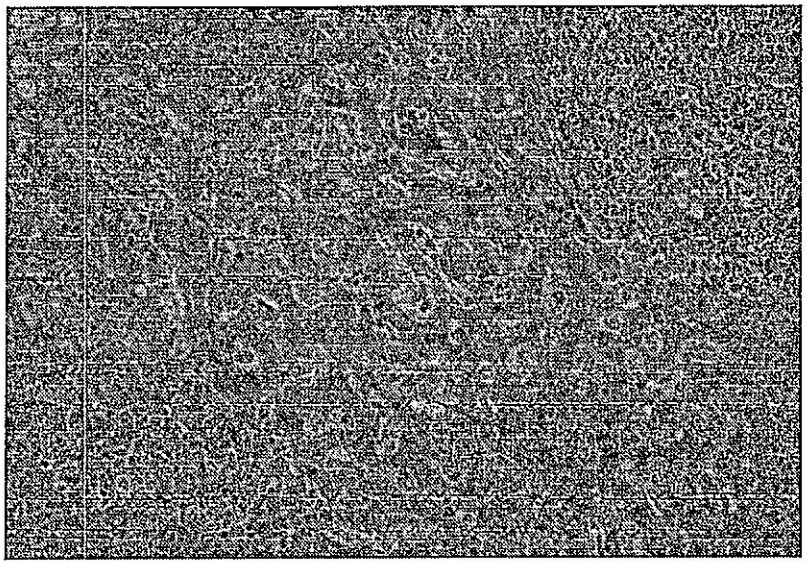

Figure 2. Canine malignant lymphoma. Accumulation of neoplastic cells in the liver. (HE, 20X)

Renal lesions were found mostly in perivascular sites in the cortex, but the renal capsule was unaffected. In one case with multicentric lymphoma, discrete tumour masses were present in the lungs as perivascular and peribronchiolar infiltrates.

Immunohistochemical demonstration of CD79, CD3 and MAC-387, showed that B-cell lymphomas were predominant in 8 of the 11 cases. T cell lymphomas were demonstrated in 3 cases. CD79 was expressed on the cells of poorly differentiated and intermediate B cell lymphomas. The pattern of expression was visible as a brown, granular, cytoplasmic precipitate on the developmental stages of B lymphocytes, but not on the plasma cells (Figure3).

The CD3 reaction was seen on mature T lymphocytes and it was visible as a brown rim in the cell periphery. In the developing stages of T lymphocytes (blast-cells) the reaction was intracytoplasmic and granular (Figure 4).

The MAC-387 reaction was very prominent on monocytes, neutrophil granulocytes and macrophages in inflammatory changes, but also monocyte and macrophage cell nests around the blood vessels in both cases of B and T lymphomas. Deposition of precipitate was granular and intracytoplasmic (Figure 5).

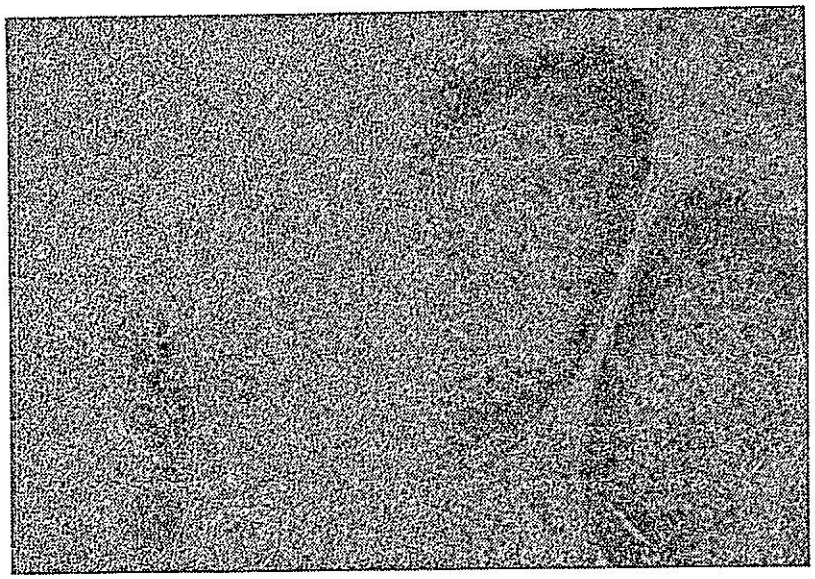

Figure 3. Canine malignant lymphoma. Expression of CD79 on the B cells of intermediate differentiated B cell lymphoma. (PAP, 20X)

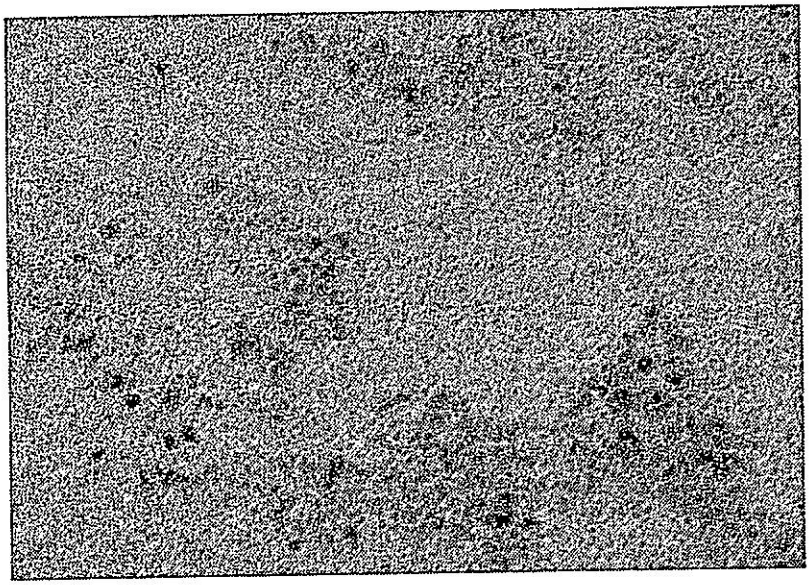

Figure 4. Canine malignant lymphoma Expression of CD3 on the T cells of a well differentiated T cell lymphoma. (DP, 40X)

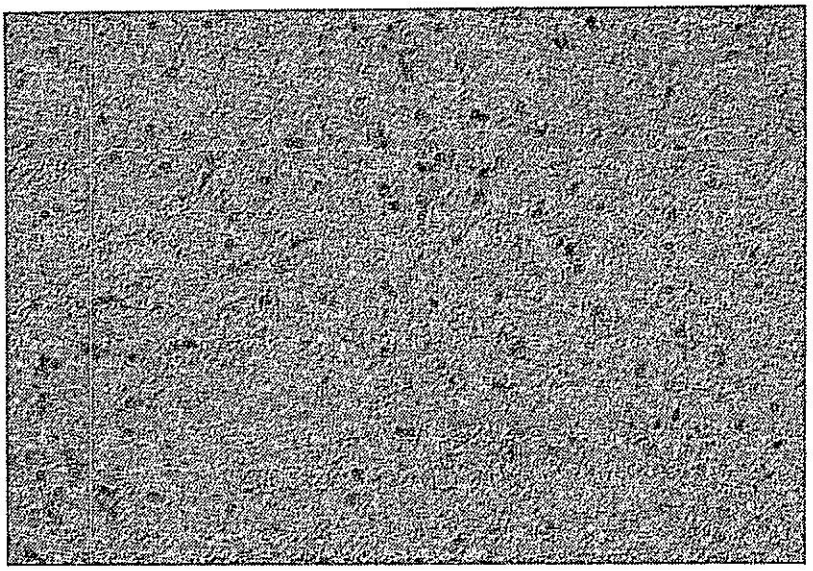

Figure 5. Canine malignant lymphoma .The MAC-387 reaction was very prominent on monocytes, neutrophil granulocytes and macrophages in B cell lymphoma. (PAP,40X)

DISCUSSION

Lymphomas are a diverse group of neoplasms of the immune system and it has been proposed that aberrations of the immune system are the cause of malignancy. An impaired humoral and cellular immune reactivity has been demonstrated in dogs with malignant lymphoma (Weiden *et al.*, 1974; Owen *et al.*, 1975).

Some breeds have an increased risk of canine NHL (boxers, Scottish terriers, Basset hound etc.), which could be related to an inherited characteristic. This is also supported by the occurrence of familial aggregation of NHL in Rotweilers (Teske *et al.*, 1994a). These results are confirmed by the breed distribution of canine lymphoma in our study. Lymphomas affect dogs of all ages, but predominantly those in middle-age. Our study is in agreement with this because the median age of the 11 dogs with lymphomas was 5,8 years and the age distribution was similar for both sexes.

The role of viruses in the aetiology of canine lymphomas is still controversial. Reverse transcriptase activity has been demonstrated in 64-79% of the culture supernatants of canine lymphomas (Onions, 1980; Armstrong *et al.*, 1982; Tomley *et al.*, 1983), but no virus could be isolated from this material. Viral influences (Epstein-Barr virus, human T-cell leukaemia virus type I), play an important role in the pathogenesis of lymphohaemopoietic neoplasm (LHN) in humans. In cats feline leukaemia virus is probably the most important cause of LHN (Kipar *et al.* 1997; Reinacher *et al.*, 1987; Aleksić-Kovačević, 1999).

In connection with non-Hodgkins lymphomas (NHL) in humans and dogs, certain herbicides, insecticides, organic solvents have been blamed, but also actinic factors (Vineis,1996).

NHL in the dog has been classified in various ways. Several types can be distinguished on the basis of anatomical location multicentric, alimentary, thymic, cutaneous and other anatomical forms (WHO,1980). By far the most common form is the multicentric or generalised lymphadenopathy. Thus, in our study 8 of 11 cases were multicentric, 2 alimentary and 1 was the cutaneous type. Many histological classifications have been used to describe lymphomas in the dog. The WHO has defined three categories: poorly differentiated, intermediate and well differentiated lymphoma (Moulton, 1990). In our study, we found that 7 of 11 tumours were poorly differentiated (lymphoblastic-LB), 3 of 11 intermediate (prolymphocytic-PLC) and 1 of 11 well differentiated (lymphocytic-LC) histological subtypes. In most of these classifications the growth pattern is an important characteristic. Most canine NHL have a diffuse or only minimally nodular growth pattern (Greenlee *et al.*, 1990; Teske *et al.*, 1994b).

Immunophenotyping of canine NHL is possible using specific canine monoclonal antibodies. With these antibodies the majority of canine NHL are classified as B cell lymphomas (Greenlee *et al.*, 1990; Teske *et al.*, 1994b). The relative number of T cell lymphomas range from 10% (Greenlee *et al.*, 1990) to 38% (Teske *et al.*, 1994b). In humans of the Western world the majority of lymphomas are of B cell origin, whereas 10 -20% are T cell lymphomas, while in Asia T cell lymphomas are more common (Lennert and Feller, 1990).

In our immunohistochemical examinations, using monoclonal mouse -anti CD79 (B cell) and rabbit-anti CD3 (T cell), we also found that the majority of lymphomas were of the B cell type (8 of 11) and only 3 of 11 derived from the T cell lineage, which is comparable with the results discussed above. MAC-387 was expressed in both immunophenotypes of lymphoid neoplasm, on infiltrating cells.

A new classification of lymphoid neoplasm, mostly based on existing terminology, was proposed by the International Lymphoma Study Group. This classification was reached through a consensus of the members and has been accepted as the Revised European American Lymphoma Classification- R.E.A.L.-Classification (Harris *et al.*, 1994). These tumours are divided into three major categories: B cell neoplasm, T cell and postulated natural killer cell neoplasm, and Hodgkins disease (Chan, 1994).

Canine malignant lymphoma (CML) is a relatively frequently occurring spontaneous tumour. It has many clinical similarities to NHL in humans. Because of increasing possibilities for diagnosis and treatment these tumours have become of considerable interest to the veterinary profession. Further studies are necessary to elucidate the aetiology and immunohistochemical classification of canine NHL.

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MORFOLOŠKA, PATOHISTOLOŠKA I IMUNOHISTOHEMIJSKA ISPITIVANJA MALIGNIH LIMFOMA PASA

ALEKSIĆ-KOVAČEVIĆ SANJA I JELESIJEVIĆ T

SADRŽAJ

Tumori limfatičnog tkiva predstavljaju jednu od najčešćih grupa tumora sa značajnim nesuglasicama u pogledu nomenklature i klasifikacije. Novu klasifikaciju limfoidnih neoplazmi ustanovila je međunarodna grupa za izučavanje limfoma i prihvaćena je pod nazivom R.E.A.L. klasifikacija. Ovi tumori su podeljeni u tri glavne kategorije: B ćelijske neoplazme, T ćelijske neoplazme i NK neoplazme i Hodkinsova bolest.

Pojava malignih limfoma pasa ustanovljena je u 11 slučajeva od 119 pasa različite rase, starosti i pola, obdukovanih na Katedri za patologiju, Fakulteta veterinarske medicine u Beogradu. Prema anatomskoj klasifikaciji uočene su tri različite forme limfoma: najčešća forma je multicentrična u 8 od 11 slučajeva, zatim alimentarna kod 2 od 11 pasa i forma kutanog limfoma kod jednog psa. Histološki su otkrivene tri različite forme limfoma: slabo diferentovani (6 slučajeva), srednji (4 slučaja) i dobro diferentovani (1 slučaj).

Imunohistohemijskim dokazivanjem CD79, CD3 i MAC-387, dokazali smo da su B ćelijski limfomi dominantni u 8 od 11 slučajeva, dok su T ćelijski limfomi u našem sekcionom materijalu dokazani u 3 slučaja.