Immunophenotypic classification of canine malignant lymphoma on formalin-fixed paraffin wax-embedded tissue by means of CD3 and CD79a cell markers

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ABSTRACT

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Canine malignant lymphoma (CML) is a common lymphoid tumour. Identification of the immunophenotype is of prognostic importance; T-cell lymphomas have a worse prognosis than B-cell lymphomas. Until recently, identification of T- or B-cell lymphomas was undertaken by means of flow cytometry or fluorescent immunocytochemistry on frozen sections. Whilst valid in the research field, these methods are impractical for routine diagnostic histopathology in CML. Commercially available CD3 antibody has been successfully employed in T-cell identification in dogs in formalin-fixed paraffin wax-embedded tissue sections, but the lack of a B-cell marker has been a hindrance until the recent introduction of a commercially available pan-B cell marker, CD79a (DAKO M7051), suitable for diagnostic application upon formalin-fixed paraffin wax-embedded material. Antibody markers to CD3 and CD79a show cross-reactivity across species lines for B cells and T cells respectively. In this group of five selected canine cases, two were identified as B-cell and the other three as T-cell lymphoma, by means of CD3 and CD79a. To the best of our knowledge application of CD79a in cases of CML has not been reported.

Keywords: Canine malignant lymphoma, CD3, CD79a immunophenotyping, paraffin wax-embedded tissue

INTRODUCTION

Neoplasms arising from lymphoid tissue in the dog are relatively common (Teske 1995a). Canine malignant lymphoma (CML) is a spontaneously developing neoplasm, characterized by the singular or multiple involvement of the liver, spleen, bone marrow and lymph nodes, and other tissues (Teske 1995b). Numerous other non-specific clinical signs including diarrhoea and weight loss may also occur (Teske 1995b). Various synonyms are used to describe lymphoid neoplasia in the dog, specifically canine malignant lymphoma, non-Hodgkin's lymphoma (NHL) and lymphosarcoma. The term in current use is canine malignant lymphoma (CML). The prevalence of CML has been reported as 13 to 24 per 100 000 dogs in two reported studies (Teske 1995a; Greenlee, Filippa, Quimby, Patnaik, Calvano, Matus, Kimmel, Hurvitz & Lieberman 1995), although it is likely that the figures have been underestimated (Teske 1995a). There is no sex predilection (Teske 1995a). Although CML may affect any age group, studies in the Netherlands suggest that there is a predilection for dogs between

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6,3 and 7,7 years of age (Teske 1995a). However, an increase in relative risk for breeds does occur; Scottish Terriers, Basset Hounds, Airedale Terriers and Bulldogs (Teske 1995a) are most at risk. Clustering has been reported, indicating the possibility of inherited, infectious and/or environmental factors playing an aetiological role (Teske 1995a). Impaired humoral and cellular immunity has been demonstrated in dogs with CML, and the question has been raised whether primary humoral and/or cell-mediated incompetence may play a role in the pathogenesis of the disease (Teske 1995a). Whether viruses play a role in the aetiology is uncertain (Teske 1995a). Environmental factors such as exposure to the herbicide 2,4-dichlorophenoxyacetic acid has also been implicated as a predisposing aetiological factor in CML (Teske 1995a).

For the classification of CML anatomical, histopathological, immunophenotypic and cytogenetic criteria are utilized (Teske 1995a; Hahn, Richardson, Hahn & Chrisman 1995; Teske, Wisman, Moore & Van Heerde 1995b; Caniatti, Roccabianca, Scanziani, Paltrinieri & Moore 1996). Although rudimentary, the anatomical classification proposed by the World Health Organization (WHO) is accepted and widely used (Teske 1995a). The most common form of CML is the generalized or multicentric form (Teske 1995a; Hahn et al. 1995). The neoplasm may be further classified by means of the clinical staging criteria proposed by the WHO (Teske 1995b). Applying a prognosis according to clinical staging is a contentious issue despite some reports having shown a favourable prognosis for dogs in stage III (generalised node involvement) rather than for those in stage IV (hepatic and/or splenic involvement) and stage V (blood and bone marrow involvement with/without systemic signs) (Teske 1995b).

Different human histopathological classifications have been used for CML (Teske 1995a; Hahn et al. 1995; Teske et al. 1995b; Greenlee et al. 1995). Two systems have been shown to best classify neoplasm in the dog; the Kiel classification and the National Cancer Institute (NCI) Working Formulation (Valli 1993; Hahn et al. 1995; Teske, Van Heerde, Rutteman, Kurzman, Moore & MacEwan 1995a; Teske et al. 1995b; Greenlee et al. 1995). Neither of these systems adequately describe extra-nodal forms and T cell-associated CML (Teske et al. 1995b). The prognostic criteria based on histopathological type according to the Kiel classification are dependent upon remission and time to relapse whereas those of the NCI Working Formulation are attributed to survival time (Teske et al. 1995a). The Kiel classification is recommended by the International Lymphoma Study Group for non-Hodgkins lymphoma (NHL) in man (Harris, Jaffe, Stein, Banks, Chan, Cleary, Delsol, De Wolf-Peeters, Falini, Gatter, Grogan, Isaacson, Knowles, Mason, Muller-Hermelink, Pileri, Piris, Ral-

kiaer & Warnke 1994). CML may be regarded as a representative model for NHL in man (Teske 1995a) and it is therefore logical that a comparable classification system should be adhered to for CML. The modified Kiel system requires immunophenotyping of B and T-cell components within the tumour (Teske et al. 1995b). In a recent prospective study by Hahn & Richardson (1995) routine clinical evaluation on the basis of human parameters of sex, age, weight, histopathologic subtype and grade, WHO clinical staging and performance status were of no prognostic significance for CML (Hahn et al. 1995). However, the histopathological classification system used in this study was the standard one, and no distinction between B- and T-cell origins was established (Hahn et al. 1995; Teske et al. 1995b). This distinction is important as Teske & Wisman (1994) reported that phenotype could not be predicted on morphological characteristics alone. In a prospective study where multivariate analysis was used, Teske et al. (1994) indicated that the T-cell phenotype is the most important independent factor associated with a poor prognosis in CML.

To date most immunophenotyping has been performed by means of fluorescent immunocytochemistry on frozen sections and fine-needle aspiration biopsy (Teske et al. 1995b; Rabanal, Ferrer & Else 1995; Caniatti et al. 1996), by using a panel of leucocyte surface markers (canine monoclonal antibodies) directed against various antigens including CD45A, Thy-1, CD49d, CD3, CD4, CD8, CD21 and anti-IgM, -IgG and -IgA. This panel of cell markers is not practical for routine histopathology on formalin-fixed paraffin wax-embedded tissue sections in two respects. Firstly, there are technical constraints as positive reactions to many of the monoclonal antibodies are inconclusive. Secondly, application of such a wide panel of cell markers is not cost effective in veterinary medicine. However, human polyclonal CD3 antiserum [DAKO A452-rabbit anti-human (DAKO A/S, Produktionsvej 42, DK-2600 Glostrup, Denmark)] has been documented as effective on canine formalin-fixed paraffin wax-embedded tissue sections and would appear to be adequate for routine histopathology as a pan-T cell marker in CML (Ferrer, Fondevila, Rabanal, Tarres & Ramis 1995; Day 1995; Nash, Scherf & Storb 1991; Teske et al. 1995b). CD3 is conserved across species lines and is an intimate intracytoplasmic portion of the T-cell transmembrane receptor (TCR) that mediates signal transduction (Jones, Cordell, Beyers, Tse & Mason 1993; Nash et al. 1991). The lack of a reliable B-cell marker that could be used on similar preparations precluded easy application of the modified Kiel classification system. Recent identification of a polypeptide encoded by the mb-1 and β29 genes, which is closely associated with the plasma membrane-associated immunoglobulins, led to the development of an antibody against mb-1 (designated CD79a at the

Fifth International Workshop on Human Leucocyte Differentiation Antigens in 1993) (Mason, Cordell, Brown, Borst, Jones, Pulford, Jaffe, Ralfkiaer, Dallenbach & Stein 1995). CD79a, therefore, seems to answer a purpose in B cells similar to that of CD3 in T cells, namely transmembrane signal transduction (Mason *et al.* 1995; Mason, Cordell, Tse, Van Dongen, Van Noesel, Micklem, Pulford, Valensi, Comans-Bitter, Borst & Gatter 1991). The monoclonal antibody JCB117 (DAKO M7051) which cross-reacts widely across species lines has been identified as a pan-B cell marker (Mason *et al.* 1995; Jones *et al.* 1993; Mason *et al.* 1991). This cell marker appears in the pre-B cell stage of development (Mason *et al.* 1995). Detection of the epitope in human B cells by JCB117 in formalin-fixed paraffin wax-embedded tissue sections may be enhanced by methods of antigen retrieval such as heating of the tissue in citrate buffer amongst others (Mason *et al.* 1995). Antibody to CD79a has been successfully applied to formalin-fixed paraffin wax-embedded tissue sections in cases of NHL and Hodgkins lymphoma in man (Mason *et al.* 1995; Mason *et al.* 1991; Korkolopoulou, Cordell, Jones, Kaklamanis, Tsenga, Gatter & Mason 1994).

This research communication reports the successful application of CD3 and CD79a cell marker systems in the immunophenotypic identification of T and B-cell CML in formalin-fixed paraffin wax-embedded tissue sections and is a preliminary report of five cases.

MATERIALS AND METHODS

Wax blocks prepared from formalin-fixed paraffin wax-embedded biopsy specimens of five cases of CML were selected from the collection at the Department of Pathology, Faculty of Veterinary Science, University of Pretoria. All of the tissues from the five cases were fixed in 10% buffered formalin, and after routine dehydration in increasing concentrations of alcohol and paraffin wax impregnation, the wax blocks were submitted to the Department of Anatomical Pathology, Medical School, University of Cape Town for immunophenotyping.

The following markers, which are used routinely in cases of human lymphomas, were selected and applied to tissue sections from each of the five cases: antibodies against CD3 (DAKO AO452); CD20; CD45R0; CD43(MB2); MT1; Cdw75 (LN-1); CD68; CD21; CD45; kappa; lambda; IgG, IgM and IgA and CD79a(DAKO M7051). All antibodies were supplied by DAKO except MB2; LN-1 and MT1.

Wax sections (15 per case) were cut at 3 μm from the blocks and mounted on APES-coated glass slides. The slides were dried overnight in an oven at 58 °C to enhance adhesion. Routine dewaxing was then carried out (15 min in zylene, and 3 min each in 100%, 96% and 70% alcohol). Endogenous per-

oxidases were blocked with 1% hydrogen peroxide (in methanol) for 15 min at room temperature. The sections were then rinsed in tap water for 5 min. The antigenic sites were then unmasked by using proteolytic enzymes such as trypsin (0,2 g in 200 mℓ Tris buffer pH 7,6–7,8, at 37 °C for 5–10 min), after which the sections were rinsed in tap water for 5 min. Alternatively, antigenic sites were unmasked by means of citrate buffer (pH 6,0), by microwaving for 10 min at high power. Kappa and lambda antigen were retrieved by means of a combination of trypsin and microwave. Slides were allowed to cool in buffer prior to staining. After blocking the slides with normal serum (goat, mouse or rabbit) at a dilution of 1:20 for 10 min at room temperature, excess serum was wiped off and the primary antibody was applied. The sections were then incubated for 60 min. All negative controls were incubated with non-immune serum from the same species as the host of the secondary antibody. After jet washing the slides in phosphate-buffered saline (PBS) (2 X 5 min) at room temperature, the secondary biotinylated antiserum (diluted 1:250) (antigoat, antimouse or antirabbit) was applied for 30 min. Following another jet washing in PBS, Peroxidase Conjugated Avidin (diluted 1:500) (DAKO PO364) was applied and incubated on the sections for 30 min. The sections were washed in PBS and then exposed to DAB [diaminobenzidine (Sigma Chemical Company, P.O. Box 14508, St Louis, MO 63178-9916)] for 5–8 min at room temperature. The sections were again rinsed (in deionised water) and counterstained with haematoxylin (monitored under microscope for adequate staining). The sections were then washed in water and "blued" in Scotts (tap water substitute) for 1 min. After rinsing them in water for 5 min, the sections were dehydrated by increasing alcohol concentrations and xylol (70%, 96%, 100% and xylol), and coverslipped, mounted in Entellan (Merck S.A. Pty Ltd, P.O. Box 259, Montague Gardens, 8001 Cape Town) (synthetic resinous mount). Staining of normal plasma cells for kappa and lambda light chains was used as positive internal control.

RESULTS

All the stained slides were examined and interpreted according to the Kiel classification and NCI Working Formulation, and it was determined whether they were positive or negative on immunohistochemistry (Table 1).

The following antibodies gave positive results in canine tissue: CD3, CD79a, CD21, CD43 (MB2), kappa and lamda light chains, IgA, IgG and IgM. The antibodies that failed to react with canine tissue were: CD45, CD20, CD45RO, MT1, Cdw75 (LN-1) and CD68. These results correlate with those shown by DAKO in their Inter-species Cross-reactivity chart,

TABLE 1   Results of classification and immunophenotyping of selected cases

| Case No.	Kiel classification	Working formulation	CD3	CD79a
1	Polymorphic centroblastic/immunoblastic	Diffuse large cell intermediate grade	–	+
2	Mycosis fungoides	Mycosis fungoides	+	–
3	High grade lymphoblastic	High grade lymphoblastic	+	–
4	High grade lymphoblastic	High grade lymphoblastic	+	– .
5	Immunoblastic with plasmacytoid differentiation	High grade large cell immunoblastic	–	+
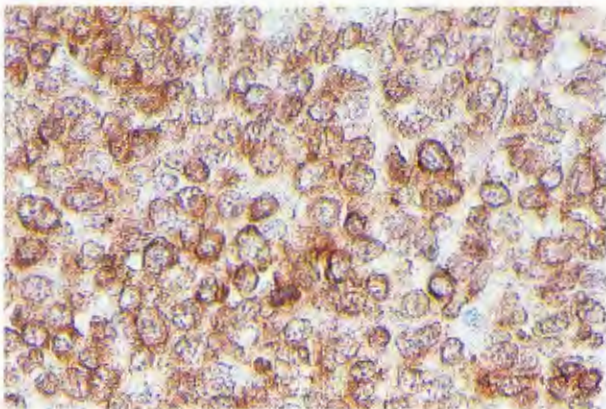
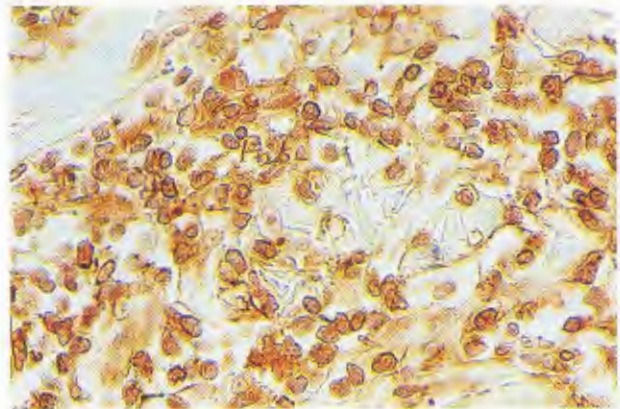FIG. 1 Positive staining pattern for CD3—perinuclear and plasma-lemmal membrane

FIG. 2 Positive staining pattern for CD79a—perinuclear and plasmalemmal membrane

with the exception of CD21, CD43 (MB2), Cdw75 (LN-1), Cd68 and CD45, which are not included in the chart.

The expected patterns of positive staining were as follows for the different markers:

CD3 and CD79a—perinuclear and cell membrane (see Fig. 1 and 2);

CD20, CD45RO, MT1 and CD45—cell membrane;

CD43 (MB2)—paranuclear (dot) and cytoplasmic (granular);

Cdw75 (LN-1)—paranuclear (dot);

CD68—cytoplasmic (granular);

CD21—irregular network staining of follicular dendritic cells;

kappa, lambda, IgG, IgM and IgA—perinuclear space/surface/cytoplasmic including perinuclear dot positivity.

The lymphoma was classified as being of T-cell origin if the neoplastic cells reacted positively with CD3 and negatively with CD79a (Fig. 1) (Table 1) and the immunoglobulins. Positive staining of the neoplastic cells with CD79a and the immunoglobulins, and negative staining with CD3 was interpreted as indicating a lymphoma of B-cell origin (Fig. 2). CD3, CD21, CD43 (MB2), Cd79a and the immunoglobulins (kappa, lambda, and IgG, IgM and IgA) reacted positively in the tissues. The other antibodies failed to cross-react with canine tissues. Strong positive staining with CD3 antibody was evident in three of the five cases; these three cases were negative with CD79a. The other two cases reacted positively with CD79a antibody and varying intensities of positive staining with the immunoglobulins, but were negative with CD3. It was concluded, therefore, that three of the lymphomas were of T-cell origin and two of B-cell origin.

DISCUSSION

Accurate histopathological classification of CML has been severely hampered in the past by the absence of a reliable pan-B cell marker, especially in view of the inability to predict the phenotype from the morphological characteristics alone (Teske et al. 1995b). The main drawback of the most widely used immunoglobulin markers is that they are not effective in identifying some cases which arose from pre-B cell clones (Mason et al. 1995). Another drawback is that several cell markers used routinely in human medicine could not be used in formalin-fixed paraffin wax-embedded tissue sections derived from CML cases, which put several immunophenotypical markers beyond the reach of the diagnostic veterinary pathologist; formalin-fixed specimens being the common specimen utilized.

The CD79a marker for B-cell CML is a practical diagnostic tool, which is readily available to the diagnostic veterinary pathologist because of its ability to

demonstrate the antigen in early pre-neoplastic and normal B cells and also because it can be used effectively on formalin-fixed tissue material. The advantages extend to veterinary research. Accurate phenotyping of CML will open up new areas of research such as determining the prevalence of non-B/non-T cell lymphomas, defining the prognosis in the different histological and phenotypic categories and the likely response of the different categories to therapeutic regimens. Furthermore, since CD3 and CD79a are apparently conserved across species lines, it is possible that these cell markers may be successfully utilized in other species.

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