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Original article

An immunohistochemical analysis of lymphocytic infiltrations in canine skin cancers

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Abstract

Lymphocytic infiltrations located in the extracellular matrix often accompany canine skin cancer. They can be characterised as an inflammatory infiltration and/or a second tumour – lymphoma. The aim of this study was an immunohistochemical analysis of a lymphocytic infiltration which accompanies spontaneous skin cancer. Twenty basal cell carcinoma, 20 non-keratinizing squamous cell carcinoma, 20 keratinizing squamous cell carcinoma and 8 sebaceous gland carcinoma samples which were accompanied by a lymphocytic infiltration and/or secondary lymphatic follicles were verified histopathologically. The expression of bcl-2, CD3, CD79α, Ki-67, MCM-3 and MCM-7 in the lymphocytic infiltration was evaluated. Four types of lymphocytic infiltrations were found: I – diffuse bcl-2^+, II – diffuse bcl-2^-, III – follicular bcl-$2^{+/-}$ where the centre was bcl-2^-, and the marginal zone of the follicles and the extrafollicular area were bcl-2^+ and IV – aggregated bcl-2^+, where the centre and periphery were bcl-2^+. The I and IV type corresponds to lymphoma, II type is non-neoplastic immune response and III type suggest reactive follicular hyperplasia. The proliferation of lymphocytes which demonstrated the expression of neoplastic markers (I and IV), suggests preneoplastic phase (pseudolymphoma) or lymphoma – the second independent tumour. A high proliferative index of the follicular blc-$2^{+/-}$ follicular infiltration indicates an increased immunological response of the host against skin cancer.

Key words: lymphocytic infiltration, skin cancer, dog, bcl-2, lymphoma

Introduction

Lymphocytic infiltration located in the extracellular matrix can often accompany skin cancer and may reduce or inhibit the proliferation of neoplastic cells. Intercellular adhesion molecule – 1 (ICAM-1, CD54) is expressed in squamous cell carcinoma (SCC) but not in basal cell carcinoma (BCC) (MacKie 2002). The ICAM-1 can interact with LFA-1 (lymphocyte function-associated antigen 1, also known as CD11a/CD18) which is commonly expressed on T cells. This interaction not only stabilizes adhesion

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but can also stimulate cytotoxic T cells to destroy the tumour cells (Basingab et al. 2016). Sometimes, the reactive lymphocytes that constitute the immune inflammatory cell reaction undergo polyclonal proliferation. This leads to the formation of an inflammatory pseudotumour with features of a T- or B-cell pseudolymphoma, which, in time, may transform into a monoclonal tumour of varying malignancy (Watanable et al. 2006). This is usually associated with a genetic instability and cytogenetic aberration such as translocation or trisomy, as well as genetic mutations in the *TP53* and *c-myc* genes (Shelton 2001). This explains the difference between a monoclonal lymphoma and an inflammatory infiltration of polyclonal lymphoid cells within tumour. Therefore, it may be assumed that the inflammatory cells can form a tumour. These cells undergo spontaneous transformation or are subjected to stimulation by intercellular "signals", such as the sonic hedgehog (SHH) protein. The SHH pathway is involved in the etiology of BCC as well as in the formation of odontogenic keratocysts, medulloblastomas, and breast carcinomas (Lupi 2007). Nongranular *MALTomata* lymphomas, also termed "secondary lymphomas", which derive from lymphocytes located *in situ* or those formed *ad hoc*, may form in a similar way in the course of autoimmunological process when cells which recognize and damage the body's own tissues do not undergo apoptosis. They may also emerge in a similar manner in immune privileged organs, such as the brain or eye (Copelan and McGuire 1995, Cupedo and Mebius 2005). In *MALTomata* a chromosomal aberration of the *BCL-10* and *MALT1* genes occurs. This interferes with the function of their corresponding proteins responsible for the formation of complexes that regulate cell apoptosis (Aster 2013).

Some cases pose a diagnostic challenge, as it is unclear whether they are affected by a reactive lymphocytic or neoplastic process. The presence of early differentiation antigens is not found in normal cells, such as CD10 present in acute lymphoblastic lymphoma, or CD1 typical for an early T-lymphocyte proliferation, which indicates a neoplastic nature of the proliferation. The antigens of other cell lines, such as CD5 (T and B1 lymphocyte markers) as well as double positive $CD4^+CD8^+$ T cells may also be present, which indicates the poorly differentiated T cell lymphoblastic lymphoma (Aster 2013). In turn, in the course of *mycosis fungoides* reduced percentage of $CD7^+$ T cells was observed (Matsuzawa et al. 2015). Reactive changes in the skin may not only contain lymphocytes, but also histiocytes, indicating progression, regression and the wax and wane of the lesion (Affolter and Moore 2000).

The aim of this study was an immunohistochemical analysis (IHC) of a lymphocytic infiltration which accompanies spontaneous skin cancer, particularly the one which forms lymphatic follicles and morphologically corresponds to malignant lymphomas. For this purpose, the expression of several antigens was tested. The B cell lymphoma 2 (bcl-2), the founding member of a family of apoptotic regulators, is considered as an important anti-apoptotic molecule which is overexpressed in malignant tumours (Correia et al. 2015).

In order to distinguish between B and T cells the CD79α and CD3 antigens were chosen. The Ki-67 and DNA replication licensing factors (MCM-3 and MCM-7) were used to determine the proliferation of cells. To date, the coincidence of epithelial (cancer) and mesenchymal (lymphoma) neoplasms in canine skin, appearing with a lymphatic infiltration, has not been described.

Materials and Methods

Materials

Sixty-eight canine skin biopsies, obtained from dogs of both genders between 5 and 11 years of age with confirmed skin cancer accompanied by lymphocytic infiltration, were used in the study. The samples previously underwent histopathological analysis by two independent pathologists, according to the WHO classification at the Division of Pathomorphology and Veterinary Forensics of the Department of Pathology of the Wrocław University of Environmental and Life Sciences. Twenty BCC (basal cell carcinoma), 20 non-keratinizing squamous cell carcinoma, 20 keratinizing squamous cell carcinoma samples and 8 sebaceous gland carcinoma samples which were accompanied by a lymphocytic infiltration and/or secondary lymphatic follicles were chosen.

Immunohistochemistry staining

Immunohistochemical analyses were conducted on 4 μm-thick paraffin sections mounted on glass slides (Dako®, Denmark), deparaffinised in xylene, and dehydrated in alcohol gradients. The EnVision FLEX Target Retrieval Solution of a low pH (Dako®, Denmark) was used for the mini-chromosome maintenance (MCM) proteins. All preparations were heated in a water bath at 96°C for 20 min. The activity of endogenous peroxidase was blocked by exposing it to the EnVision™ FLEX Peroxidase-Blocking Reagent for 10 min. The following primary antibodies were applied: rabbit monoclonal anti-human MCM-3

(clone EP202, dilution 1:50, Novocastra), MCM-7 (clone DCS 141.1, 1:50, Novocastra), monoclonal mouse anti-human Ki-67 (clone MIB-1, 1:100, Dako®), bcl-2 (clone 124, 1:50, Dako®), CD3 (clone CT-3, 1:100, Dako®) and CD79α (clone Ig 225, 1:50, Novocastra). The lymphoma from lymph node was used as the positive control for blc-2 staining, and the normal lymph node was used as negative control. The section of normal lymph node was used as the positive control for lymphocytes (CD3, CD79α) and proliferation markers. The slides were incubated at room temperature for 20 min. They were subsequently washed in the EnVision™ Wash Buffer. Next, EnVision™ FLEX/HR SM802 visualisation system reagents were applied, and the slides were incubated at room temperature for 20 min. The IHC reaction was developed using 3,3 – diaminobenzidine tetrahydrochloride (DAB) in the EnVision™ FLEX DAB$^+$ Chromogen (Dako®). Finally, the slides were rinsed in distilled water, counterstained with haematoxylin, dehydrated in alcohol gradients, passed through xylene and sealed.

Antigen expression analysis

The slides were subjected to image analysis with a computer connected to an Olympus BX53 optic microscope (Olympus, Japan) equipped with an Olympus Color View IIIu digital camera (Olympus, Japan). All measurements were taken using cell ^{A} software (Olympus Soft Imaging Solution, Germany). A four-level scale was used to determine the intensity of the expression of the studied antigens: 0-5% cells (negative reaction, –) and positive reactions: 6-25% (+), 26-50% (++), 51-80% (+++) and >80% (++++). All cases were reviewed by two independent pathologists.

Statistical analysis

The results obtained were analysed statistically using Statistica 12.5 software (StatSoft Polska, Cracow, Poland). Statistical significance of the data was assessed using Student's t-test or the Mann-Whitney U test, depending on the normality of data distribution. A value of $p<0.05$ was considered statistically significant. Spearman's correlation analysis was used to assess the relationship between variables.

Results

Basing on anti-bcl-2 staining and morphology, four types of lymphocytic infiltrations were distinguished to accompany canine skin cancer, regardless of the tumour subtype, gender and age of the animal (Fig. 1):

I – diffuse bcl-2 positive infiltration (++++) – 32 dogs. The CD3$^+$ (++) or CD79α$^+$ (++) cells predominated in the view field depending on the case.

II – diffuse bcl-2 negative infiltration (9 dogs). The CD3$^+$ cells (++) predominated in the view field, but scattered CD79α$^+$ cells (+) were also present.

III – follicular bcl-2$^{+/-}$ infiltration where the centre was bcl-2 negative, and the marginal zone of the follicles as well as the extrafollicular area were bcl-2 positive (++++) – 21 dogs. The germinal centres of follicles were dominated by CD79α$^+$ and CD3$^-$ cells, whereas those of the mantle and extrafollicular area were CD3$^+$ (+++) and CD79α$^-$. Single histiocytes, plasmocytes and macrophages localised in the germinal centres of follicles, corresponding to the structure of pseudolymphoma, were encountered during the histological analysis.

IV – aggregated bcl-2 positive (++++) infiltration, which morphologically corresponds to a malignant lymphoma (6 dogs). The centre and periphery of the infiltration were bcl-2 positive. Clusters of cells that formed *ad hoc* were usually composed of small CD3$^+$ cells. This corresponds to a neoplastic proliferation that occurs in a primary, non-Hodgkin SALT/SIS – T cell type lymphoma. In some cases a combination of CD79α$^+$ centroblasts and centrocytes with a high mitotic index, and containing single cells undergoing apoptosis was observed.

The expression of the MCM-3 and MCM-7 proliferative antigens revealed a very high positive correlation (correlation coefficient $r=0.94$, $p<0.05$) (Fig. 2). Ki-67 and MCM-3 as well as Ki-67 and MCM-7 also showed a high positive correlation ($r=0.63$, $p<0.05$ in both cases). The expression of these antigens was most intense (+++/++++) in the germinal centres of the follicles. It was found to be expressed at a level of + in the marginal area of lymphatic follicles, the extrafollicular area and in groups I and II.

Discussion

The tumour-associated leukocytes are the immunological response of an organism against neoplastic cells, as well as concomitant bacterial infections or stromal regression processes (i.e. ulcerations). Cytotoxic T lymphocytes (CTL, CD8$^+$) and natural killer (NK) cells kill the tumour cells (Copelan and McGuire 1995), while the T helper lymphocytes (Th, CD4$^+$) are involved in neoplastic cell destruction, by activating NK cells, CTLs and macrophages. In some malignant tumours, tumour-infiltrating lymphocytes

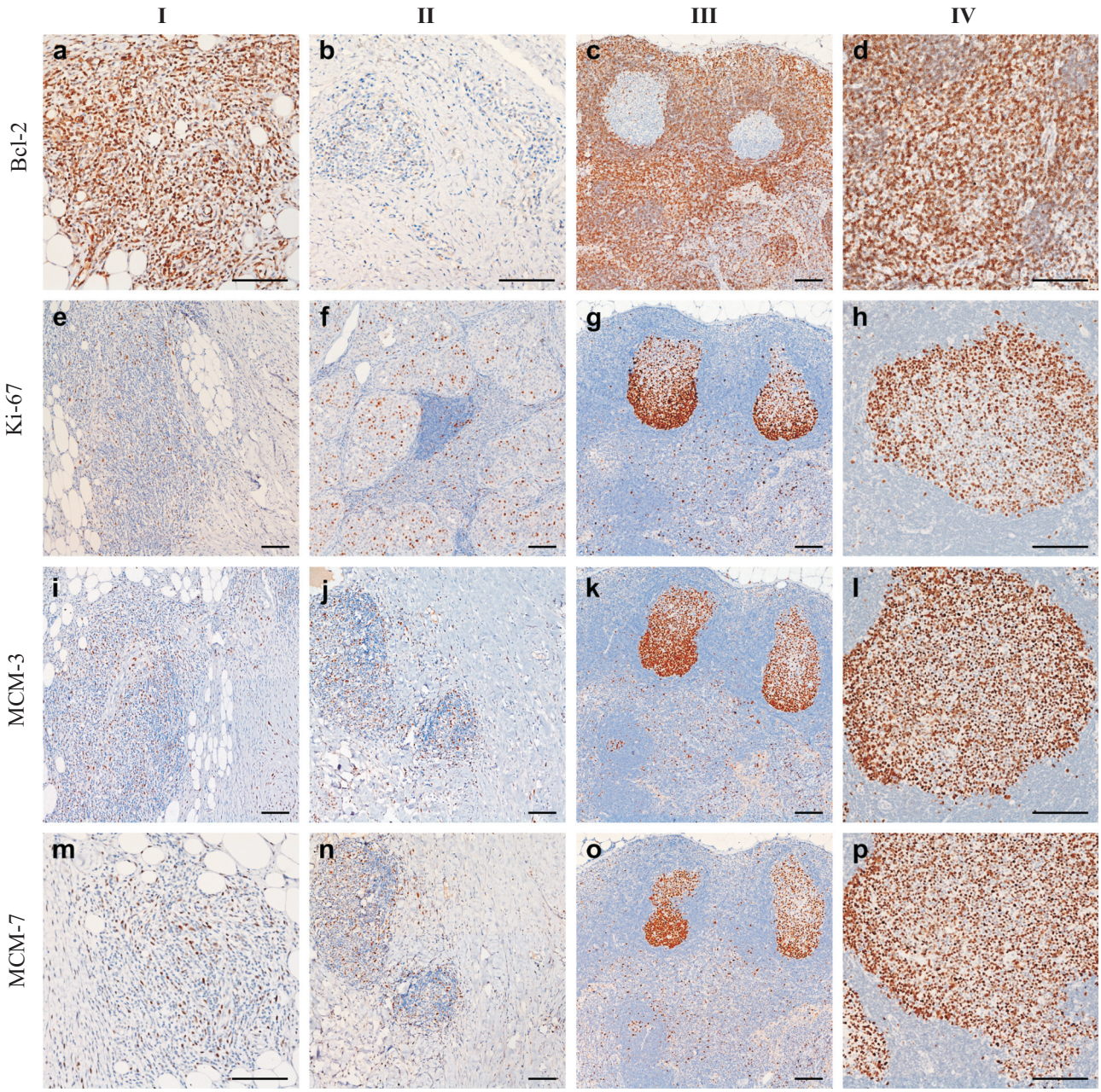

Fig. 1. Examples of bcl-2, Ki-67, MCM-3 and MCM-7 expression in lymphocytic infiltrations: I – diffuse bcl-2^{+}, II – diffuse bcl-2^{-}, III – follicular bcl-2$^{+/-}$, IV – aggregated bcl-2^{+}; scale bar = 100 µm.

(TIL) and lymphokine-activated killer (LAK) are also found (Hsiao et al. 2004). LAK are IL-2 stimulated mononuclear cells where the CD3-NK cells dominate, but TCR/CD3 T cells are also produced (García-Muñoz et al. 2015). They also stimulate the accumulation of fibroblasts, which inhibit in turn the growth of the tumour by isolating it from the surrounding healthy tissue.

Occasionally, lymphatic tissue resembling follicles of secondary lymphoid organs is found in the skin. It may be able to synthetize antibodies *in situ,* as is seen in patients with psoriasis and rheumatoid arthritis (Cupedo and Mebius 2005). Lymphoid follicles may also be present in portal areas in the course of *hepatitis chronica persistens* and bronchiogenic cysts (*cystis bronchiogenes*) in human patients (Aster 2013). To date, it is unclear whether this process is initiated by an ongoing inflammatory response. Lymphoid follicles that form *ad hoc* and lymphocytic infiltrations may also accompany mammary carcinoma in female dogs (Madej 2015). These infiltrations are reactive, and might proliferate to form usually aggregated T-cell lymphoma. The immunophenotype of this proliferation is characterised by the expression of CD3^{+},

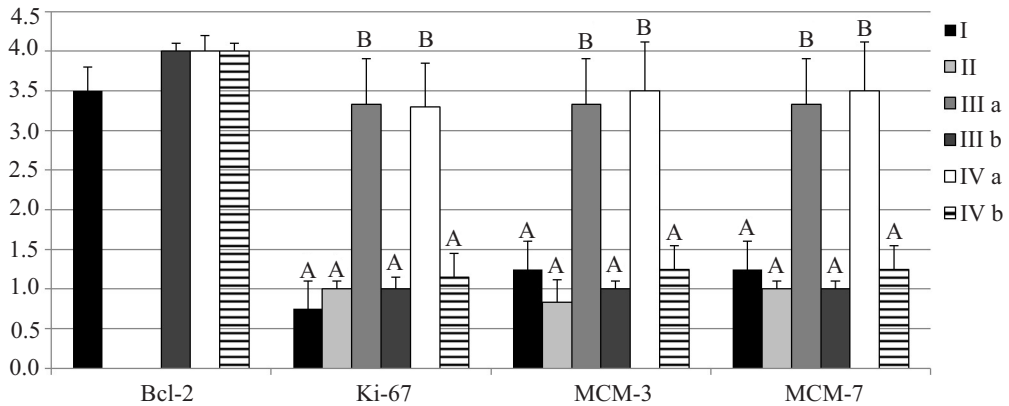

Fig. 2. The level of bcl-2, Ki-67, MCM-3 and MCM-7 (mean + SD) antigen expression on a 1-4 scale in the lymphocytic infiltrations: I – diffuse bcl-2^+, II – diffuse bcl-2^-, IIIa – germinal centre of the follicle bcl-$2^{+/-}$, IIIb – follicular wall and inter-follicular bcl-$2^{+/-}$ infiltration, IVa – centre of the aggregated infiltration bcl-2^+, IVb – periphery of the aggregated infiltration bcl-2^+. Significant difference between A and B in the given group ($p<0.05$).

$CD10^+$, $CD5^{+/-}$ antigens, a lack of CD43 and CD79α antigens expression, and, in contrast to humans, no response to CD20 and cutaneous lymphocyte-associated antigen (Madej 2015). Our study indicate that lymphocytic diffuse bcl-2 positive or negative infiltration, as well as aggregated strongly blc-2^+ infiltration may accompany skin cancer, irrespective of its type. Pseudolymphomas are formed from a polyclonal cell infiltration that includes lymphocytes, histiocytes, eosynophils and plasma cells. In such proliferations the germinal centres (GCs) of lymphoid follicles and tingible-body macrophages are also clearly visible (Aster 2013). Sometimes a process of inflammatory atopic dermatitis and neoplasia are closely intertwined, as is the case in mycosis fungoides. In such cases the neoplastic process revealed late, which is explained by depletion of Th1 lymphocytes followed by loss of immunological response (Shelton 2001, Aster 2013). The etiology of pseudolymphoma proliferations is complicated and may be drug-induced (penicillins, nitrofurantoin, atenolol, captopril), or caused by skin trauma or lymphoid contact dermatitis (balsam of Peru, nickel). It may also be caused by sunlight (actinic reticuloid) or persistent nodular arthropod-bite reactions, such as those of scabies mites and ticks, which transmit *Borrelia burgdorferi* – and trigger borelial lymphocytoma (*lymphadenitis benigna cutis*) (Shelton 2001, McKie 2002).

In our study, a high proliferative index of follicular cell infiltrations was noted. This may indicate an intense local immunological response against skin cancer in dogs as observed in follicular blc-$2^{+/-}$ infiltrations. In some cases, the lymphoid infiltration resembled a primary T-cell non-Hodgkin's lymphoma. According to the WHO-EORTC (European Organization for Research and Treatment of Cancer) classification from 1997, it corresponded to human cutaneous lymphoma (Watanable et al. 2006). Follicular lymphomas show a strong bcl-2 expression within the GCs, unlike reactive cells that are bcl-2 negative. Therefore, the presence of bcl-2 antigen in dogs allows to distinguish the GCs in lymphomas from non-neoplastic B-cells. In the present study, two types of non-diffuse infiltrations were observed. The follicular bcl-$2^{+/-}$ infiltration suggests reactive follicular hyperplasia where the GC were blc-2^-, but mantle zone and interfollicular cells were blc-2 positive. The aggregated bcl-2^+ infiltration corresponds to T-cell lymphoma or follicular lymphoma. There were also diffuse bcl-2^+ infiltration observed. Similar, entirely diffuse neoplastic infiltration in human skin is called a diffuse follicle centre lymphoma (Aster 2013). The diffuse blc-2 negative infiltration was classified as reactive infiltration of the inflammatory cells.

The strongly bcl-2 positive centrocytes were also observed in GCs of the lymph nodes, whereas most of the remaining lymph nodes showed bcl-2-negative follicular hyperplasia (Cong et al. 2002). Clinical study suggests, that nearly half of such cases represent homing and early colonization of reactive GCs by follicular lymphoma (Cong et al. 2002). Other cases might represent follicular lymphoma at the earlier stages of development, or a preneoplastic lesions, which require a second genetic hit to neoplastic transformation.

This phenomenon usually is known as in situ follicular lymphoma which has been described in normal lymph nodes from clinically healthy patients, normal lymph nodes from patients with a prior or concurrent diagnosis of follicular lymphoma, lymph nodes involved by other hematolymphoid malignancies, and lymph nodes examined during the evaluation of non-hematolymphoid neoplasms (Montes-Moreno et al. 2010, Roullet et al. 2010, Carbone et al. 2011a,b, Carbone and Gloghini 2011, Nybakken et al. 2016).

MCM is a protein that supervises the initiation and elongation process during DNA synthesis. In the G1 phase, inactive proteins are added during the above mentioned processes and then are transformed to enzymatically active helicase in the S phase (Aparicio et al. 1997, Musahl et al. 1998). Therefore, the expression of the MCM protein and the Ki-67antigen is only present in proliferating cells. The concentration of Ki-67 rises in the G1 phase and peaks at M phase. It is not detected in the G0 phase (Scholzen and Gerdes 2000). Our study revealed that the expression of proliferative factors such as Ki-67, MCM-3 and MCM-7 was higher in the GCs of bcl-$2^{+/-}$ follicular infiltrations and in the centre of the bcl-2^{+} aggregated infiltrations. The expression of proliferative markers in lymphomas and benign disorders from skin biopsies was previously reported by Ralfkiaer et al. (1986).

There is a multistage induction mechanism of the T-cell lymphoma in humans. Th1 lymphocytes produce IFN-γ, which stimulates a high expression of ICAM-1 on keratinocytes. Lymphocytes that possess the LFA-1 adhesion molecule on their surface are recruited to the epithelium (McKie 2002). After the formation of the tumour, the number of Th2 lymphocytes that produce IL-4 rises. This inhibits IFNγ synthesis and, consequently, leads to a decrease in the expression of ICAM-1, a decline of epidermatotropism, and an increase in the lymphoma cell infiltration within the tumour. Neoplastic Th2 cells constitutively produce increased amounts of IL-4, IL-5 and IL-10, which decreases the T-cell response to antigens and mitogens, and reduces the activity of NK and LAK cells (McKie 2002). The development of T cell lymphoma exhibits some analogy with atopic dermatitis, where the above mentioned interleukins are also produced. However, Th2 cells are cancerous in lymphoma (McKie 2002).

In the present study, the proliferation of lymphocytes in canine skin cancer which demonstrated the expression of neoplastic markers, suggests preneoplastic phase (pseudolymphoma) or lymphoma. In preneoplastic phase, a clone of cells typical of a malignant SALT/SIS type of lymphoma forms a second independent tumour with distinct autonomous characteristics. It can be classified as a synchronous tumour and a member of multiple primary malignancies (according to the International Agency for Research on Cancer from 1991), because it develops independently of the cancer formed in the same place (Rebhun and Thamm 2010). The present results indicate that both tumours can be accompanied by a lymphocytic reaction of a non-cancerous phenotype most likely associated with the presence of the cancer. The presence of this reaction suggests that the tumour has been already identified by the immune system of the host.

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