Abstract. In order to investigate the immune mechanisms involved in regression of canine cutaneous histicytoma (CCH), major histocompatibility complex (MHC) class-II immuno-expression and the number of T- and B-lymphocytes and macrophages were analyzed in 93 cases of CCH. MHC class-II was also studied in 16 cases of CCH by immunoelectron microscopy. All tumors expressed MHC class-II, and two major staining patterns were identified: focal juxtanuclear cytoplasmic staining and rim-like staining along the cell periphery. The MHC class-II labelling pattern and T- and B-lymphocyte infiltrates were associated with tumor regression. In regressing lesions, MHC class-II molecules shift to the cell surface and an increase of both T- and B-lymphocytes were noted. The increasing expression of MHC class-II molecules on the cell surface could be a significant factor for the onset and progression of tumour regression.

Canine cutaneous histiocytoma (CCH) is a benign neoplasm of Langerhans cells. The disease affects mainly young dogs and often regresses spontaneously (1-4). CCH has similarities to some forms of human Langerhans cell histiocytosis (LCH). Nevertheless, LCH has distinct clinical behaviours in dogs and humans. In contrast to dogs, only a limited number of lesions undergo regression in humans (5-7) and other lesions can disseminate and be potentially fatal (8-10). CCH could be regarded as a unique model to study human LCH and to understand the immune mechanisms involved in tumour regression.

Complete or partial regression of various tumour types has been documented both in man (11-14) and animals (15-17). In CCH, regression has been associated with an initial infiltration of T-helper cells (CD4+), followed by increased expression of T-helper 1 (Th1) cytokines and recruitment of antitumour effector cells (18). However, the factors that determine the onset of regression in canine histiocytomas are still not well understood. The aim of the present study was to clarify a possible role of major histocompatibility complex class-II (MHC-II) in regression of CCH.

Materials and Methods

Tissue processing and tumour classification. Ninety-three cases of CCH and 10 normal canine skin samples, obtained from the University of Trás-os-Montes and Alto Douro Histopathology Laboratory archives in four years, were included in this study. Each sample was re-examined by two independent pathologists (IP and AA) in order to confirm the diagnosis, according to the criteria of the World Health Organization International Histological Classification of Tumors of Domestic Animals (19). Histiocytomas were also grouped according to the degree and patterns of intra-tumoural lymphocytic infiltrate into four groups, as described previously (20), representing various stages of tumour regression.

Immunohistochemistry. For immunohistochemical studies, 3-μm sections of all samples were cut and mounted on silane-coated slides. The antibodies used were: mouse anti-human Human Leukocyte Antigen DR (HLA-DR, α-chain) (MHC-II, D33, 1:50; Dako®, Carpinteria, California, USA), rabbit anti-human CD3 (1:100; Dako®, Carpinteria, California, USA), mouse anti-human CD79a (HM57, 1:50; Dako®, Carpinteria, California, USA) and mouse anti-human leucocyte protein L1 (calprotectin) (MAC387, 1:100; AbDSerotec®, Oxford, UK). The detection of the antigens was carried out by the streptavidin-biotin-peroxidase complex method, with a commercial detection system (Ultra Vision Detection System, Lab Vision Corporation, Fremont, California, USA), following the manufacturer’s
instructions. Antigen retrieval was carried out by microwave treatment with sodium citrate buffer at pH 6, with microwave irradiation (750 W) for 3 x 5 min. Antigen retrieval was not used before staining for MHC-II. Immunoreactivity was visualized by incubation with 13,3-diaminobenzidinetetrahydrochloride (DAB) at 0.05% with 0.01% H2O2 as the final substrate for 5 min. After a final washing in distilled water, the sections were counterstained with Mayer’s haematoxylin, dehydrated, cleared and mounted. The primary antibody was replaced with phosphate buffered saline (PBS) for negative controls, and positive controls consisted of sections from normal canine skin, skin adjacent to neoplastic tissue and canine lymph node lymphocytes.

Quantification of immunolabelling. Positivity was indicated by the presence of distinct brown labelling. Immunoreactivity was evaluated blindly by two pathologists using a semi-quantitative method. For CD3+ and CD79+ lymphocytes and for MAC387+ macrophages, the percentage of cells exhibiting a positive immunohistological reaction was estimated according to the number of positive cells in relation to the total number of the cells present in the histiocytoma (~0%；+1-10%；++: 11-25%；+++: 26-50%；++++: >51%). MHC class-II antigen was evaluated in the tumour cells as ~0%；+1-10%；++: 11-25%；+++: 26-50%；++++: >51% of positive tumoural cells. The labelling pattern for MHC-II antigen was also evaluated according to the site of the positive reaction as membranous (more than 50% of cells with membranous reaction) or cytoplasmic (more than 50% of cells with cytoplasmic reaction).

MHC class-II immunolectron study. The MHC-II immunoelectron study was performed on 16 samples fixed in formalin (four tumours of each histological group), using the pre-embedding method, following the standard technique used at the Institute of Histology and Embryology of the Medicine Faculty, Coimbra, Portugal. The immunocytochemical labelling was performed on thick sections (50 μm), prior to embedding in plastic resin. The reagents were similar to those used in the process of immunohistochemistry for observation by light microscopy. After staining with uranyl acetate, dehydrating and embedding, thin sections were observed in a transmission electron microscope (100S, Jeol®, Tokyo, Japan) at 40-60 kV.

Statistical analysis. Associations between histological groups and positivity for CD3, CD79, MAC387 and MHC-II immunolabelling were performed using the χ2 test, with the SPSS system version 12.0 (SPSS Inc., Chicago, Illinois, USA). A value of p<0.05 was considered significant.

Results

Tumour classification. According to the number and distribution of lymphoid infiltrates (20), 15 cases were classified as group I, 15 cases as group II, 42 as group III and 21 as group IV.

Immunohistochemistry. CD3+ cells were present in all tumours (93/93) while CD79+ lymphocytes were absent from 26 cases. In all lesiions, we observed more CD3+ T-lymphocytes than B-cells. In groups I and II, both B- and T-lymphocytes, when present, were identified at the bottom of the tumours. In group III and IV, they were found diffusely between neoplastic cells, being the major cell population in group IV histiocytomas. Statistical analysis revealed a significant increase of CD3+ T- and CD79+ B-lymphocytes from group I to groups II, III and IV (p<0.001; Table I).

A positive reaction for MAC387 was shown in intravascular monocytes and neutrophils present in ulcerated areas and in keratinocytes from the granulosum and corneum stratus of epidermis and follicle. A variable number of MAC387+ macrophages were observed between neoplastic cells and lymphocytes, but no association with histological groups was seen (Table I).

The MHC-II antigen was expressed in different cells in CCH. In lymphocytes, the labelling was represented by a small rim in the cell periphery. Exudate macrophages and monocytes exhibited diffuse cytoplasmic staining. Epidermal Langerhans cells exhibited a cytoplasmic-positive reaction in cell processes.

In tumoural cells of CCH, MHC-II was present diffusely in all lesions (+++). Two major patterns of immunolabelling were observed: a cytoplasmic (diffuse or focal juxtanuclear) and a membranous reaction. In 22 tumours (23.7%) the labelling was predominantly cytoplasmic, and in 71 (76.3%) the positivity was predominantly membranous.
The labelling pattern varied significantly among the different histological groups ($p<0.001$; Table I). In group I tumours, most of the tumoural cells displayed a cytoplasmic reaction (Figure 1), except for a small number of cells localised in the tumour periphery, close to the lymphocytic infiltration. In group II, we observed both patterns, with 8 out of 15 tumours having a predominantly membranous reaction. Generally, in tumoural cells of the epidermal surface and in the center of the lesions, the immunoreaction was predominantly cytoplasmic, diffuse or with a juxtanuclear pattern, and in the cells at the tumour base the immunolabelling was membranous. In tumours of group III, the labelling pattern was predominantly membranous (Figure 2), except for a small number of cells near the epidermal surface. In group IV, the labelling was exclusively membranous in all cases.

Similarly to the associations observed in the histological groups the pattern of MHC-II reaction in CCH tumoural cells were statistically associated with the presence of both CD3+ T-lymphocytes and CD79+ B-lymphocytes ($p<0.001$), but not with MAC387+ macrophages ($p=0.085$).

**Immunoelectron microscopic study.** Immunoelectron microscopic studies, using the pre-embedding method, did not allow for a perfect preservation of cells and tissues. However, in all samples, the results were complementary to those obtained by light microscopy. A cytoplasmic pattern reaction, diffuse or perinuclear (Figure 3), was predominant in tumours with a low lymphoid infiltrate (group I and II) and MHC-II antigens were membrane bound, associated with endoplasmic reticulum and Golgi complex, and in vesicles, some of which had a multivesicular appearance (Figure 4). MHC-II antigens were also present along the cell surface of the tumoural cells, in the tumoural cell membrane (Figure 5), more commonly in group III and IV tumours.

**Discussion**

MHC-II antigens were detected in all CCH studied here, as described in previous work (21, 22). However, as far as we know, this is the first study that includes all histological groups and an immunoelectron study. The previous study that reported a relationship between the immunoexpression of MHC-II antigen and CCH regression (22) did not include group III and IV tumours, which limits the conclusions that can be done. As described by Kippar et al. (22), we observed two major patterns of labelling: intracytoplasmic and...
Peripheral in the cell membrane. By immunoelectron methods, we distinguished a positive reaction for MHC-II in the cytoplasm (sometimes diffuse, but most often membrane-bound, located in the perinuclear area) and in the cytoplasmic membrane of tumoural cells. These findings seem to reflect different stages of the mechanism of biosynthesis and organization of the MHC molecules during the immune response mediated by Langerhans cells and dendritic cells. In immature dendritic cells, most MHC-II molecules are localized intracellularly in MHC-II compartments in storage vesicles, endosomes and lysosomes, the MHC-II-enriched compartments (23). These compartments, also identified in Langerhans cells (24, 25), have variable morphology: a multivesicular appearance or an intermediate form with internal vesicles and concentric membrane arrays (26, 27). In mature dendritic cells, the compartments of MHC-II are rearranged and the MHC-II molecules are efficiently transported to the cytoplasmic membrane (24, 28). In fact, histiocytoma cells in CCH seem to mimic normal dendritic cells in that the capacity to stimulate T-lymphocytes is related to their ability to regulate the distribution of MHC-II molecules intracellularly (29). Moreover, the MHC-II molecules in the juxtanuclear area of CCH tumoural cells may represent storage granules containing newly-synthesized MHC-II molecules which have not yet been fused with endosomes (22, 23). The presence of MHC-II molecules in the cell membrane, also described in human Langerhans cell histiocytosis studies (30), could represent a T-cell-activating ‘mature’ functional state of CCH, a significant factor for the onset and progression of tumour regression (22). In tumours of groups III and IV, the cytoplasmic reaction was reduced, possibly because after maturation, the synthesis of MHC-II molecules decreases (27).

In the present study, the regression of CCH appears to be associated not only with the shift of MHC-II molecules in tumoural cells, but also with the infiltration of T-lymphocytes, as previously described (18). Optimal T-cell activation requires two distinct signals. The first signal is mediated by MHC-restricted, antigen-specific triggering of the T-cell receptor complex and the second by an antigen-independent mechanism, termed co-stimulation. On dendritic cells, MHC-II molecules present peptides to immunologically-naive CD4+ T-cells, which become activated only when their antigen-specific T-cell receptors interact with cognate MHC-II peptide complexes on mature dendritic cells (31). The second signal is provided through surface receptors, such as CD28. CD28 ligands of the B7 family are expressed constitutively by professional antigen-presenting cells, including dendritic cells, monocytes and activated B-cells (32, 33).

Other molecules may be involved in CCH regression. A decrease in or lack of E-cadherin expression in regressing CCH has been reported in previous work (34, 35), suggesting a maturation of the tumoural cells. Beyond the activation of tumour-specific lymphocytes, CD4+ T-cells possess further antitumour functions in the recruitment of non-specific effector cells such as macrophages, which can exert additional tumouricidal activity by synthesis of reactive metabolites (18). However, our study does not support this hypothesis. The intervention of dermal dendritic cells in regression is also suggested in histiocytoma (22).

This study also suggests a role for B-lymphocytes in CCH regression. This may be the result of complex cross-talk mechanisms between dendritic cells and lymphocytes (36-38),
and probably, as in the immune response, involves multiple integrated signalling pathways by different molecules. Full understanding of these processes can only be achieved by performing more dynamic analyses of local immunity.

In conclusion, this study strongly suggests that tumoural cells in CCH appear in an immature state in early lesions and undergo a maturation process characterized by an increased expression of MHC molecules in the cell periphery. In regressing lesions, these cells may differentiate towards a mature phenotype and might themselves interact with lymphocytes. The shift of MHC molecules in the cell periphery seems to be a significant factor for the initiation and progression of CCH regression.

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