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Major histocompatibility class II expression in the normal canine cornea and in canine chronic superficial keratitis

D. L. Williams

Department of Clinical Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge CB3 OES, England, UK

Address communications to:

David L. Williams

Tel. and Fax: +44 (0)1223 232977
e-mail: doctordlwilliams@aol.com/
dlw33@cam.ac.uk

Abstract

Objective To compare the expression of major histocompatibility complex (MHC) class II antigen in the corneas of normal dogs and dogs affected with chronic superficial keratitis (CSK).

Methods MHC class II expression was determined in frozen sections of normal canine cornea and cornea from lesions of CSK by immunohistochemistry using a monoclonal antibody directed against the canine MHC class II molecule. Langerhans cell phenotype was determined morphologically and by histochemical determination of ATPase activity. To determine the influence of gamma interferon on expression of MHC class II molecules by corneal cells, corneal explants were cultured with the cytokine and MHC class II expression determined as above.

Results Numerous MHC class II-expressing cells were demonstrated within the stroma and epithelium of the normal corneal limbus and conjunctival epithelium while very little MHC class II expression was detected in the central region of normal canine cornea. In limbal and conjunctival epithelium, cells expressing MHC class II antigen showed ATPase activity, suggesting that they were Langerhans cells. Corneas from dogs with CSK showed MHC class II expression associated with stromal cells, some of which exhibited a dendritic morphology while most were lymphocytic. Corneal epithelial cells within the lesion also aberrantly expressed MHC class II. Corneal explants expressed MHC class II to varying degrees after differing periods of incubation with the cytokine gamma interferon.

Conclusions While the normal central cornea has little MHC class II expression, aberrant expression occurs in CSK, associated with secretion of gamma interferon by infiltrating CD4-expressing lymphocytes. Although this change is likely to be a secondary feature of the CSK lesion, increased MHC class II expression may play a part in perpetuating the corneal inflammation seen in the disease.

Key Words: autoimmunity, chronic superficial keratitis, dog lymphocyte MHC class II antigen

INTRODUCTION

Canine chronic superficial keratitis (CSK) is a nonulcerative inflammatory disease of the superficial corneal stroma occurring most frequently, but not exclusively, in the German Shepherd dog. Previous studies have attempted to implicate an infectious cause, without success. The condition develops as a progressive bilateral, mononuclear cell infiltration from the temporal region of the limbus into the superficial corneal stroma.[1] A progressive fibroplasia and vascularization ensues, often leading to involvement of the anterior stroma across the entire expanse of the cornea. Blindness typically results in untreated cases. Clinical cases appear to be more severe both at higher altitudes and in hotter climates, suggesting an actinic modulation of the disease.

The histopathologic inflammatory characteristics of the disease,[1,2] the predominance of CD4-expressing lymphocytes in the lesions,[3] and the response to both topical steroids and cyclosporine[4] lend support to the hypothesis that CSK is an autoimmune disease. Previous work has also demonstrated production of gamma interferon by infiltrating lymphocytes.[3] Given the previously reported association between this cytokine and up-regulation of major histocompatability class II antigen expression, the current study aimed to determine the

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expression of the MHC class II antigen in the normal and CSK-affected canine cornea, and to investigate up-regulation of MHC class II antigen expression in the canine cornea when incubated with gamma interferon.

MHC class II expression was first demonstrated *de novo* on the thyroid epithelium of patients with autoimmune thyroiditis.[5] Subsequent experiments showed that these cells could present thyroid autoantigens to autoreactive T lymphocytes. These observations led to considerable speculation regarding the role of MHC class II expression in autoimmune disease. Aberrant expression of MHC Class II has been implicated in the pathogenesis of a variety of autoimmune diseases, including primary biliary cirrhosis, diabetes mellitus and rheumatoid arthritis.[6] In the cornea, MHC class II expression has been reported in herpetic keratitis,[7] although the role of such changes has not been fully defined.

The central cornea had previously been reported to demonstrate a low level of MHC class II expression.[8] This was considered to be an important factor in the apparent immune-privileged immune status of the transplanted cornea from non-MHC matched donors.[9] In fact it is MHC class II expression in the epithelial layer which is absent in the central cornea; stromal keratocytes do express MHC class II antigen in the normal cornea and may be involved in antigen presentation in the inflamed cornea.[10]

Investigation of MHC class II expression in the normal cornea and aberrant expression in the CSK lesion is an important component in the immunologic characterization of CSK. Monoclonal antibodies to canine MHC class II have thus been used to evaluate the expression of this antigen in normal and CSK-affected canine cornea, and ATPase staining has been used as a marker for Langerhans cell phenotype.

MATERIALS AND METHODS

Normal tissues were collected from five ophthalmoscopically normal adult dogs after euthanasia by barbiturate overdose. Biopsy material from five CSK-affected dogs was obtained during therapeutic superficial keratectomy as described previously.[3] These animals had been free from immunosuppressive therapy for a minimum of 4 weeks, and all had active disease at the time of surgery. Within 30 min of removal, unfixed specimens were embedded without fixation, in cryoembedding gel (OCT Tissue Tek, Sakura, Japan) and snap frozen in isopentane cooled in liquid nitrogen. Frozen sections were cut at 5 μm and mounted on slides coated in 3 amino propyl triethoxysilane.[11] Sections were rehydrated in 0.05 M tris buffered saline at pH 7.3, and incubated at room temperature in 1 : 10 normal rabbit serum for 30 min. Sections were then incubated for 2 h with neat IgG2a culture supernatant containing a rat monoclonal antibody specifically directed against canine class II major histocompatibility antigens.[12] Sections were then rinsed for three periods of 5 min in 0.05 M tris buffered saline at pH 7.3. Sections were then incubated for 1 h in 1 : 50 fluorescein isothiocyanate conjugated rabbit antibody directed against rat IgG (Serotec, Oxford, UK). Sections were rinsed for three periods of 5 min in 0.05 M tris buffered saline at pH 7.3 and mounted in Fluorostab (Eurodiagnostics, East Grinstead, UK). Control sections were incubated with a first layer of either 1 : 10 normal rabbit serum or a neat culture supernatant rat IgG monoclonal with a noncanine specificity (mouse IgG). All sera were diluted in 0.05 M tris buffered saline with 1% BSA and 0.1% sodium azide. Sections were examined on an Olympus BHS microscope (Harpenden, UK) equipped for epifluorescence photomicroscopy.

Determination of ATPase, as described by Berman and France,[13] was performed on tissue cryoembedded and sectioned as detailed above. Assessment of coexpression of antigens was achieved by using adjacent sections cut at between 3 and 5 μm. Sections were fixed in cold 4% formaldehyde, buffered in cold 0.08 M sodium cacodylate for 10 h and rinsed in 0.25 M tris buffered saline. They were then incubated in a solution of 0.5% $MgSO_4$, 0.12% $PbNO_3$ and 0.04% adenosine 5′-triphosphate disodium salt (Sigma Chemical Co, Poole, UK) at 37 °C for 20 min and subsequently rinsed thoroughly in 0.25 M tris buffered saline. Following incubation in dilute ammonium sulfide for 5 min at room temperature the sections were rinsed in distilled H_2O before mounting.

Corneal explants were obtained from eyes of ophthalmically normal dogs euthanased for a variety of terminal conditions. Full-thickness discs of central cornea were harvested in a sterile manner within 10 min of death using a disposable, 5-mm biopsy trephine (Kruuse, Odense, Denmark). Explants were cultured in Dulbecco's modified essential medium supplemented with penicillin and streptomycin (Sigma Chemical Co.) and containing recombinant murine gamma interferon (Sigma Chemical Co.). The cytokine was produced in *Escherichia coli* with a gene identical in sequence to that of murine gamma interferon (personal communication, Toray Industries Inc., Yokyo, Japan). Activity was 8.3×10^4 i.u./mg and a stock solution of 1×10^3 iμ/mL had previously been determined to have less than 0.05 ng/mL endotoxin as measured by the *Limulus* amebosyte lysate test.[14] Explants were cultured in 0, 200, 500 and 1000 iμ/mL gamma interferon for 6, 12 and 48 h and also in 10 μL/mL recombinant murine Fc gamma from the same cell source, as a control.

RESULTS

Negative controls for the immunofluorescence staining showed only faint nonspecific background staining. The normal canine central cornea showed no MHC class II expression by any epithelial cells, and only by stromal keratocytes (Fig. 1). The normal limbus showed moderate numbers of stromal cells that expressed MHC class II, as did dendritic cells in the limbus (Fig. 2) and conjunctival epithelium (Fig. 3).

Corneas affected with chronic superficial keratitis showed increased immunofluorescence at several sites. At the border of the inflammatory infiltrate, increased numbers of subepithelial cells were stained, as well as a small number of

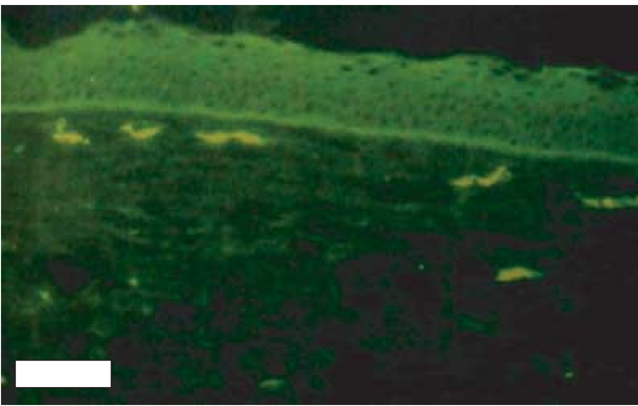

Figure 1. Immunocytochemical staining of the MHC class II positive stromal cells of the normal central canine cornea. Bar = 50 μm.

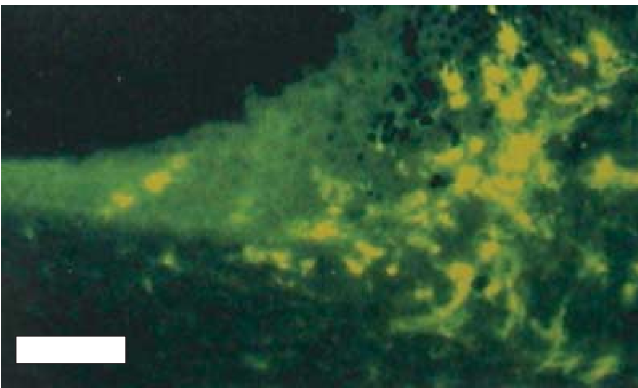

Figure 2. Immunocytochemical staining of the MHC class II positive stromal cells in the normal peripheral cornea and limbus. Bar = 100 μm.

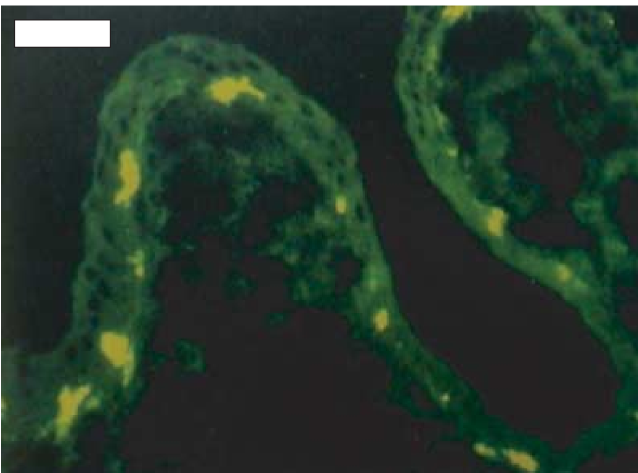

Figure 3. Immunocytochemical staining of MHC class II positive dendritic cells in the conjunctival epithelium. Bar = 50 μm.

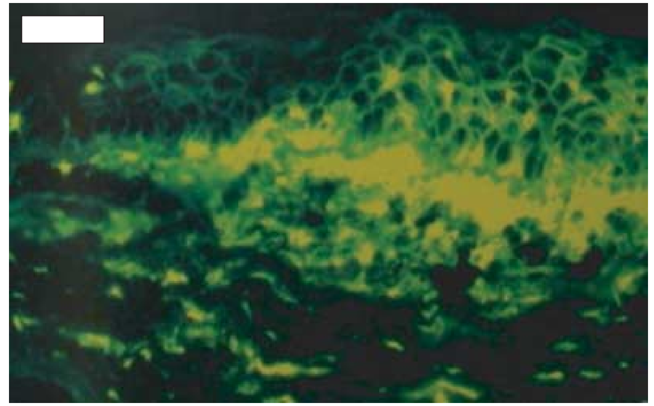

Figure 4. Immunocytochemical staining of MHC class II positive cells in the junctional area between normal and diseased cornea in an affected dog. Bar = 50 μm.

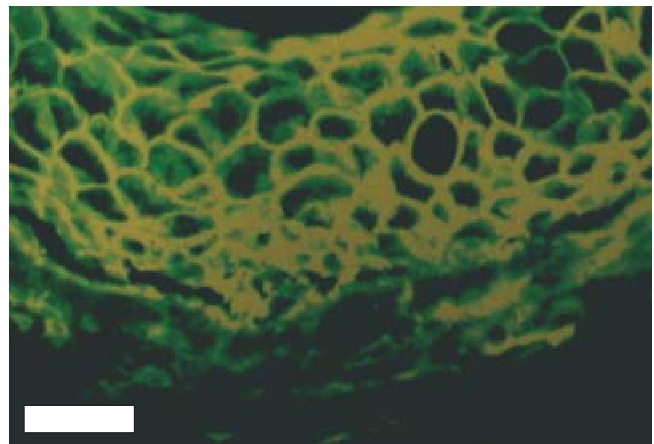

Figure 5. Immunocytochemical staining of MHC class II positive stromal and epithelial cells in the most severely affected area of the CSK lesion. Bar = 25 μm.

Figure 6. ATPase staining of Langerhans cells at the normal limbus with characteristic dendritic phenotype. Bar = 25 μm.

intraepithelial cells (Fig. 4). These cells were characterized by dendritic morphology on phase contrast light microscopy. In the more developed areas of the lesion the majority of epithelial cells were stained as were substantial numbers of subepithelial cells (Figs 4 and 5). Intensity of staining and number of cells stained increased from the edge of the infiltrate to the center of the lesion. A small number of epithelial cells stained positively immediately in front of the subepithelial

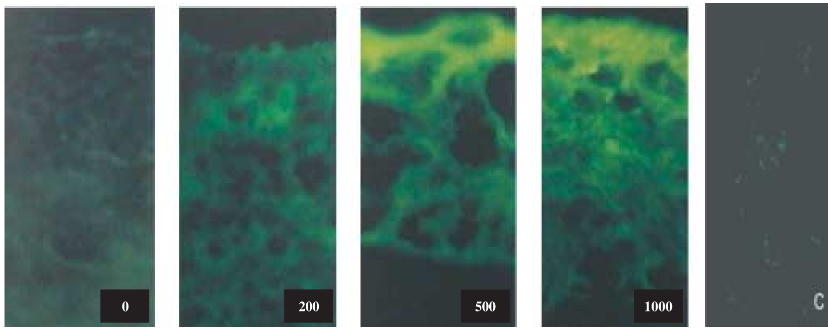

Figure 7. MHC class II expression induced in normal corneal epithelial cells incubated with murine g interferon at concentrations of 0, 200, 500 and 1000 i.u./mL and incubated with murine IgG at 10 μg/mL as a control (C). Faint staining in the control was also seen in cornea stained without using anti-MHC class II antibody.

infiltrate (Fig. 4) while all epithelial cells stained strongly in the center of the lesion (Fig. 5).

Neither epithelial nor stromal staining for ATPase was detected in the normal central cornea, while moderate numbers of epithelial cells were stained at the normal limbus and in the bulbar conjunctiva. Numerous darkly stained cells were observed throughout the lesion in sections of cornea affected by CSK stained for ATPase, while blood vessel walls were also stained. These keratocytes showed a dendritic rather than lymphoid morphology (Fig. 6) which, together with their ATPase staining, strongly suggested that they were Langerhans cells. Staining of consecutive sections with hematoxylin and eosin showed that, while other sections in more advanced regions of the lesion were characterized by pigmentation, no pigmented cells were seen in the analogous areas in sections examined by immunofluorescence. The dark staining noted in these cells was a result of the ATPase staining technique rather than corneal pigment.

Normal canine corneal explants cultured in the presence of murine gamma interferon showed an increase in expression of the MHC class II antigen by epithelial cells, between concentrations of 0 and 500 i.u./mL as demonstrated in Fig. 7, but not by stromal keratocytes. Culture with higher concentrations of gamma interferon (500–1000 iμ/mL) did not increase the level of MHC class II expression but rather led to the development of areas of epithelial degeneration. Culture with 10 μg/mL murine Fc gamma did not result in induction of MHC class II expression, with the slight staining observed also seen in tissue stained without anti-MHC class II antibody.

DISCUSSION

The lack of MHC class II expression in central corneal epithelium has for some time been considered an integral part of the mechanism of immune privilege in the cornea.[9] Langerhans cells, which express MHC class II and are capable of presenting antigen to lymphocytes, have been reported at the normal corneal limbus and within the conjunctival epithelium of many species. However, such cells were considered absent or present only at very low densities, in the central cornea of the adult.[8] The immunohistochemical results reported here confirm a similar distribution of these cells in the epithelium of the dog, and are corroborated by a comparable distribution of staining for ATPase, specific for Langerhans cells.[15] A small number of stromal keratocytes in the normal central dog cornea do, however, express MHC class II molecules but do not contain ATPase. Research has confirmed this stromal distribution of MHC class II-expressing keratocytes in the mouse.[10] A heterogenous population of dendritic cells has been defined, some expressing MHC class II antigen throughout life and others not expressing the antigen until stimulated by an inflammatory milieu.[16–19] These cells have been shown to migrate to draining lymph nodes during inflammatory responses in the cornea.[20] Unfortunately, in the dog only a limited number of monoclonal antibodies directed against leukocyte antigens are available, rendering further phenotypic investigation of canine stromal dendritic cells types impossible.[21] Similarly, recombinant canine gamma interferon was not available and thus a murine substitute was used. While there is no published material showing that canine tissue responds to murine interferon gamma, the highly conserved nature of the molecule across species barriers[22] suggests that its use in this study is acceptable.

A migration of Langerhans cells into the central cornea has been noted in a number of experimental studies and clinical diseases.[23] Their presence in the cornea is an important predisposing factor in clinical and experimental graft rejection. Furthermore, removal of these cells by UV irradiation,[24] or incubation at high oxygen tensions or in hypothermic conditions,[25] prolongs graft survival. Migration of Langerhans cells into the central cornea can be induced by IL-1 production in the cornea.[26] Not only can MHC class II positive Langerhans cells increase in number in diseased corneas, however, but also epithelial cells themselves can be induced to express this antigen.[27] This finding is mirrored by the observations reported here.

While aberrant class II expression was originally thought to be a primary event in autoimmune conditions, it is now considered to be secondary manifestation of other initiating mechanisms. Nevertheless, current opinion suggests a role for such expression in the accentuation or prolongation of an immune-mediated attack. Gamma interferon induces MHC class II expression on a variety of cells that normally lack this antigen, including corneal epithelium, as shown here. Furthermore, intraperitoneal administration of gamma interferon in mice results in enhanced class II expression on

corneal stromal cells in the absence of centripetal migration of limbal Langerhans cells.[28] It has been demonstrated that gamma interferon is produced by the lymphocytes migrating into the CSK-affected cornea;[3] this source would explain the genesis of aberrant MHC class II expression in epithelial cells in the center of the lesion.

In the current study, interpretation of the immunofluorescence staining in these corneas was more complicated because, in contrast to many other species, canine B lymphocytes and activated canine T lymphocytes express MHC class II constitutively.[29] Hence, the positively staining cells in the subepithelial zone of the normal and diseased tissue may be lymphocytes. Previous histopathologic work has shown that lymphocytes are the predominant inflammatory cell in these lesions.[1,2] Morphologically, however, a proportion of the MHC class II-expressing stromal cells appear dendritic and they do not stain with a monoclonal antibody directed against CD3 using an indirect immunocytochemical technique (data not shown). This suggests that MHC class II positive stromal cells observed here are either migrating Langerhans cells or stromal fibroblasts.

The part which Langerhans cell migration to the central cornea, and aberrant MHC class II expression on epithelial cells play in the pathogenesis of the putative immune-mediated lesion of CSK is at present unclear. However, even if MHC class II expression is not instrumental in the genesis of inflammatory lesions in CSK, it is likely to play an important part in prolonging the inflammatory process in these corneas. It may also allow the development of an autoimmune reaction to antigens normally present in the canine cornea.

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