

# Histological and immunohistochemical evaluation of canine chronic superficial keratitis

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## SUMMARY

Chronic superficial keratitis (CSK) is an inflammatory disease of the canine cornea, characterised by infiltration of leucocytes into the anterior corneal stroma. The present study describes a quantitative histomorphometric analysis of the cell types infiltrating the corneal stroma in this disease. Infiltrating cells were quantified in samples taken at superficial keratectomy and processed for routine histology. Further characterisation of lymphocyte phenotypes was achieved by immunohistochemistry performed using a panel of monoclonal antibodies recognising canine lymphocyte CD antigens. Lymphocytes expressing the CD4 antigen were found to be the predominant infiltrating cell types in the CSK lesion. A significantly smaller number of lymphocytes expressed the CD8 antigen. The CD4/CD8 ratio was consistently above 2 and rose to above 4 at the advancing border of the lesion. A proportion of lymphocytes were shown by immunohistochemistry to contain gamma interferon. This study forms a basis for work further evaluating the cytological events central to the development of this spontaneous potentially auto-immune corneal disease.

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CHRONIC superficial keratitis (CSK) is a canine corneal inflammatory disease, occurring predominantly, but not exclusively in the German shepherd dog. It manifests clinically as fibrovascular plaque with a distinct border, surrounded by a translucent halo consisting of initial invading cells together with some corneal oedema. The lesion is self limiting in some dogs while in others it progresses, if untreated, to affect the entire cornea. The developing lesion is characterised histologically by a lymphocytic infiltration into the superficial corneal stroma. This initial phase is followed by invasion of a mixed inflammatory cell population together with vascularisation and, in many cases, pigment deposition. The clinical appearance of CSK has been described previously (Bedford and Longstaffe 1981, Slatter et al 1977). Investigations of the presence or absence of cellular responses to corneal antigens have yielded conflicting results (Campbell et al 1975, Peiffer 1978). Previous studies have described the histopathological appearance of CSK qualitatively (Bedford and Longstaffe 1981, Slatter et al 1977) and one group has reported immunohistochemical evaluation of immunoglobulin deposition in the lesion (Eichbaum et al 1986). Quantification and immunophenotypic characterisation of cellular infiltration of the cornea in CSK has not, however, previously been described.

It is suggested here that quantitative evaluation of cell phenotype from the margin of the progressing cellular infiltrate to the centre of the lesion provides a valuable insight into the changes occurring through time as the lesion progresses. In other immune-mediated diseases, sequential samples must be taken in order to follow the course of the disease. This may involve the sacrifice of several animals at different time points in an experimental model, as has been undertaken in experimental autoimmune uveoretinitis (Liversidge and Forrester 1988, Brown et al 1989). Alternatively the developing lesion may be sampled during the course of clinical

disease as has been undertaken in rheumatoid arthritis (Rooney et al 1988). CSK, however, presents a unique opportunity for defining cellular changes in the progression of a disease in a single biopsy sample. In addition, the superficial nature of the CSK lesion renders it possible to remove the entire lesion while maintaining corneal integrity and simultaneously restoring normal visual function. Material for histopathological analysis can thus be obtained from clinical cases without compromising the welfare of the animal.

Monoclonal antibodies specifically directed against canine leucocyte surface antigens are available through the Canine Leucocyte Antigen Workshop (Cobbold and Metcalfe 1994). Several groups have applied these antibodies to the immunocytochemical and immunohistochemical evaluation of canine immunemediated diseases (Williams, 1997) and here their use in determining lymphocyte phenotype in the CSK lesion is reported.

## MATERIALS AND METHODS

Therapeutic superficial keratectomy was performed on five dogs affected with CSK, three being pure-bred German shepherd dogs and two German shepherd dog crosses. Normal cornea was obtained from five ophthalmoscopically normal dogs euthanased because of non-ocular and non-immune mediated disease. Representative samples of corneal tissue removed at surgery were bisected with one half processed for routine histology while the other was used for immunohistochemistry. Samples for routine histology were fixed in 10 per cent neutral buffered formal saline. Tissue for immunohistochemistry was snap frozen in isopentane cooled in liquid nitrogen.

Formalin-fixed tissues were processed for routine histology. Three- $\mu$ m serial sections were stained with Mayer's

**TABLE 1: Antibodies from the Canine Leucocyte Antigen Workshop**

CLAW number	Originating laboratory	Antibody dilution	Canine specificity
8	Cobbold, Cambridge, UK	1:200	CD4 <sup>+</sup> lymphocytes and neutrophils
56	Gebhard, Raleigh, USA	1:150	CD4 <sup>+</sup> lymphocytes and neutrophils
1	Cobbold, Cambridge, UK	1:75	CD5 pan lymphocyte marker
71	Davis, Pullman, USA	1:150	CD5 pan lymphocyte marker
11	Cobbold, Cambridge, UK	1:400	CD8 <sup>+</sup> lymphocytes
55	Gebhard, Raleigh, USA	1:250	CD8 <sup>+</sup> lymphocytes
9	Cobbold, Cambridge, UK	1:100	pan CD45 leucocyte common antigen
112	Alejandro, Miami, USA	1:50	restricted epitope of CD45
13	Cobbold, Cambridge, UK	1:250	restricted epitope of CD45
–	Fuller, Miami, USA	1:200	$\gamma$ -interferon

haematoxylin and eosin. Slides were viewed under an Olympus BH2 light microscope equipped with an eyepiece counting graticule. Total cell number at given distances from the lesion border was determined using the counting graticule in ten fields at  $\times 400$  magnification. Populations of lymphocytes, plasma cells, macrophages, neutrophils, fibroblasts and fibrocytes were thus enumerated.

Five- $\mu\text{m}$  cryostat sections of snap-frozen unfixed tissue were cut at  $-20^\circ\text{C}$  on a Bright Instruments cryostat. All antibody dilutions and buffer washes used phosphate-buffered saline at pH 7.3, containing  $100\text{ mg l}^{-1}$  of both  $\text{CaCl}_2$  and  $\text{MgCl}_2$  (PBS). Sections were hydrated with PBS and incubated for 30 minutes with 1:10 normal goat serum in PBS at room temperature. Following aspiration of the 1:10 normal goat serum, tissue sections were incubated with primary antibodies at room temperature for 1 hour. Primary monoclonal antibodies raised in rat or mouse are listed in Table 1 and were provided through the Canine Leucocyte Antigen Workshop (Cobbold and Metcalfe 1994). Murine monoclonal antibody specific for canine  $\text{IFN-}\gamma$  was provided by Dr Fuller, Miami, Florida (Fuller et al 1994). Appropriate dilution of each primary antibody was determined by assessing staining of frozen sections of canine lymph node at a range of dilutions. Sections were rinsed for 15 minutes in three changes of PBS and then incubated for 1 hour in secondary antibody.

The secondary antibodies used in the present study were fluorescein isothiocyanate-conjugated goat anti-rat IgG and rhodamine isothiocyanate-conjugated goat antimouse IgG and IgM (Sigma Chem Co., Poole, UK.). Secondary antibodies were used at a dilution of 1:40. Following incubation with secondary antibody, sections were rinsed with PBS for three periods of five minutes. For co-localisation of two antigens, rat and mouse primary monoclonal antibodies were combined at appropriate dilutions in the primary antibody incubation. Sections were then incubated with a similar combination of anti-mouse rhodamine-conjugated and anti-rat fluorescein-conjugated secondary antibodies. Following the final PBS rinse, sections were mounted in Fluorostab mounting medium (Eurodiagnostica, East Grinstead) and observed with an Olympus BH2 microscope equipped for epifluorescence and with an eyepiece counting graticule. Immunohistochemically stained cells were counted in ten  $\times 400$  fields at varying distances from the edge of the lesion boundary. Each section was compared with a corresponding control that had been incubated with secondary fluorochrome-conjugated antibody and a sheep anti-mouse Ig primary monoclonal antibody. Such sections were consistently unstained.

## RESULTS

No invading leucocytes were detected in the normal corneas evaluated. A dense inflammatory cell infiltrate was demonstrated in the superficial stroma in CSK. Deeper stroma was almost entirely devoid of inflammatory cells. Lymphocytes were the initial infiltrating inflammatory cells and remained the predominant infiltrating cells throughout the lesion. Fibroblasts and fibrocytes were found at significantly higher numbers in the developing lesion than in normal cornea. Plasma cells, macrophages and small numbers of neutrophils were also found later in the developing lesion, the latter cells particularly associated with the developing corneal neovascularisation. A representative histological section of the developed lesion is shown in Fig 1. Cell counts from histological sections of corneas taken from five animals are shown in Figs 2 and 3.

The majority of non-fibroblastic cells in the lesion expressed the CD5 antigen on immunohistochemistry, demonstrating them to be lymphocytes. Anti-canine CD3 antibody was not available at the time of this study and thus anti-CD5 antibody was used as a lymphocyte marker although it is recognised that CD5 is not a fully pan-T-cell epitope. Immunohistochemical co-localisation of CD4 and CD8 antigens in tissue sections demonstrated that CD4-expressing T lymphocytes predominate throughout the lesion. For all investigations the substitution of an irrelevant antibody (sheep antimouse IgG) gave unstained sections. Fig 4 documents numbers of CD4-expressing and CD8-expressing cells through the developing lesion, showing that the CD4:CD8 ratio was always substantially greater than unity and was significantly higher at the advancing border of the lesion. Immunohistochemical localisation of  $\text{IFN-}\gamma$  is shown in Fig 5. A significant number but not the majority of lymphocytes within the CSK lesion contained this cytokine. The well demarcated intracytoplasmic staining observed suggests that staining is specific for  $\text{IFN-}\gamma$  produced in the cells rather than cytokine present throughout the lesion. Clearly this assumption can only be verified by *in situ* hybridisation, but at the time of this investigation *in situ* canine cytokine probes were not available. The proportions of lymphoid cells containing  $\text{IFN-}\gamma$  through the lesion are demonstrated in Fig 6.

## DISCUSSION

CSK is an inflammatory disease of the superficial canine corneal stroma. This study demonstrates that the CSK lesion is characterised by a lymphocytic infiltrate predominantly

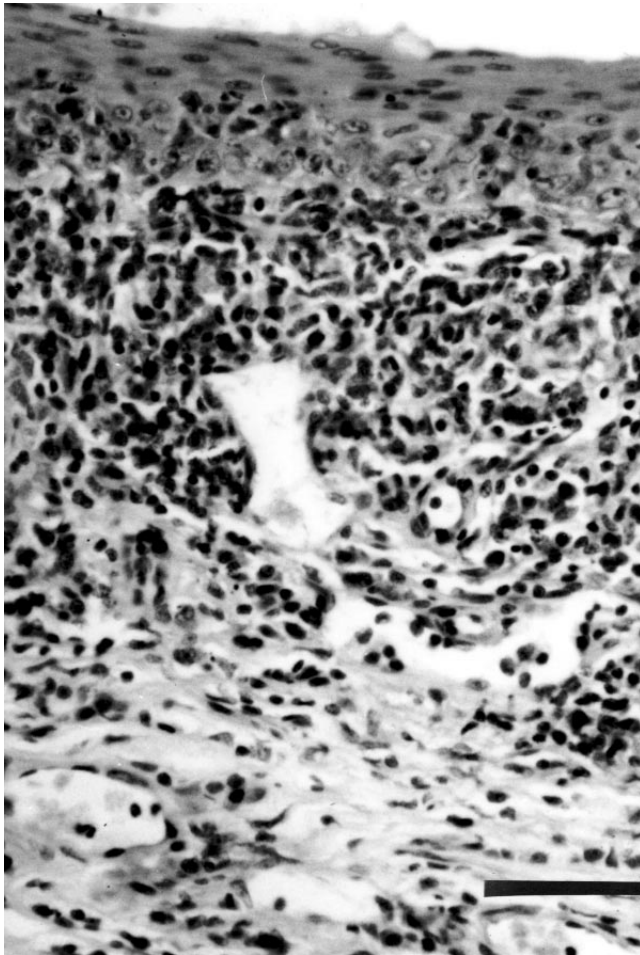


FIG 1: Representative histological section of chronic superficial keratitis. Mononuclear cell infiltrate is present in the anterior stroma with some cells invading into the epithelium. Formalin fixed tissue, haematoxylin and eosin stain. Bar=50  $\mu$ m

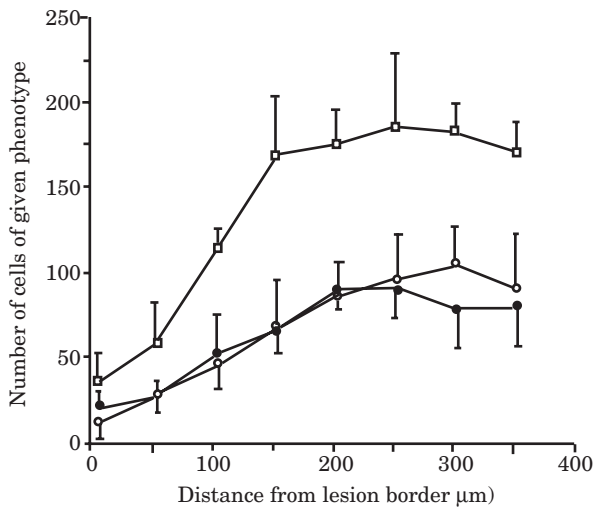


FIG 2: The mean number and SEM of fibroblastic cells in 10 high power fields across the chronic superficial keratitis lesion in five dogs. —●— fibroblasts; —○— fibrocytes; —□— fibroblasts + fibrocytes

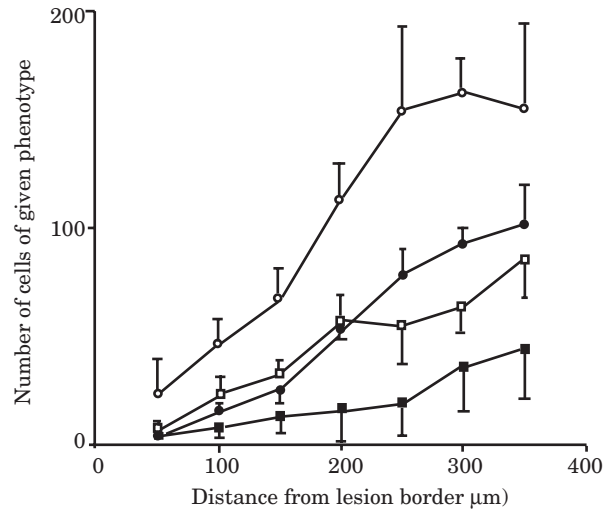


FIG 3: The mean number and SEM of polymorphonuclear leucocytes, Lymphocytes, plasma cells and macrophages in 10 high power fields across the chronic superficial keratitis lesion in five dogs. —■— polymorphonuclear leucocytes; —○— lymphocytes; —●— plasma cells; —□— macrophages.

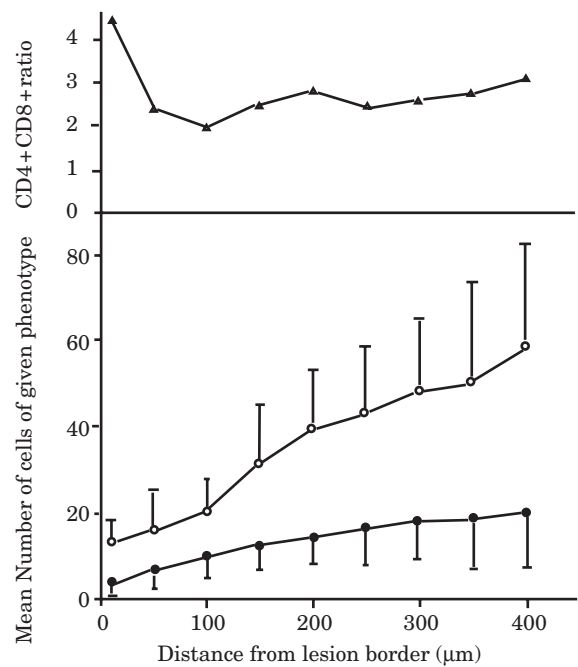


FIG 4: The mean number and SEM of CD4-expressing and CD8-expressing cells and the CD4:CD8 ratio in 10 high power fields across the chronic superficial keratitis lesion in five dogs. —○— CD4<sup>+</sup> lymphocytes; —●— CD8<sup>+</sup> lymphocytes

composed of CD4-expressing T-helper cells. Low numbers of CD8-expressing cytotoxic or suppressor lymphocytes were present throughout the lesion. At the advancing border of the developing lesion all invading lymphocytes expressed the CD4 antigen. Macrophages, plasma cells and neutrophils were shown to invade the corneal stroma at later stages in the development of the lesion. The latter of these three cell types was primarily associated with the neovascularisation seen in the fully established lesion although CD8-expressing lymphocytes and plasma cells were seen

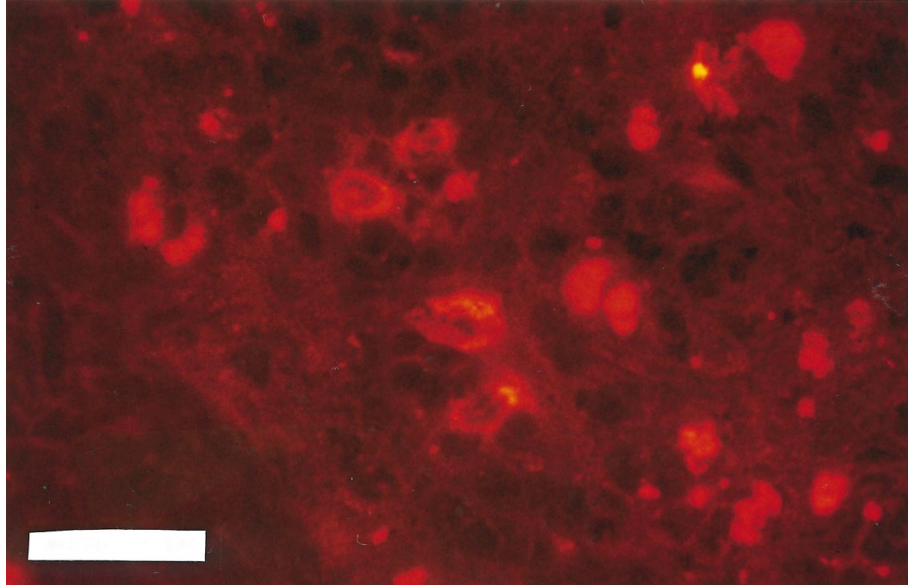


FIG 5: Immunohistochemical localisation of IFN- $\gamma$  in cytoplasm of a proportion of lymphoid cells. (snap-frozen tissue, bar=10  $\mu$ m).

throughout the developed lesion, although at lower numbers than CD4-expressing mononuclear cells.

In the dog the CD4 antigen is expressed on the surface of neutrophils as well as lymphocytes (Moore et al 1992). Only a small number of polymorphonuclear leucocytes occur in the lesion, however, and these can readily be differentiated from lymphoid cells by their nuclear shape, clearly demonstrated in histological sections and also visible by phase contrast microscopy in unstained cryostat sections.

The predominance of CD4-expressing lymphocytes in the lesion mirrors the cellular characteristics of a number of autoimmune conditions. Lymphocytes expressing this antigen predominate in experimental autoimmune diseases, such as experimental autoimmune uveitis (Liversidge and Forester 1981) and experimental allergic encephalitis (Spiram et al 1982) together with human diseases such as autoimmune thyroid disease and rheumatoid arthritis (Janosy et al 1981). The predominance of the CD4-expressing lymphocyte in CSK suggests that an autoimmune pathogenesis may account for the disease.

A significant proportion of lymphocytes in the CSK lesions investigated here contained IFN- $\gamma$  and that this proportion increased through the development of the lesion.

Murine CD4-expressing cells have been subdivided into two populations based on their cytokine production: Th1 cells produce IL2 and IFN- $\gamma$  while Th2 cells produce IL4 and IL5 (Mosman et al 1986). This demarcation is less clear in the rat and human with cells occurring which cannot be placed in either the sub-population. Different diseases are characterised by different Th1 or Th2 populations: T lymphocytes in human multiple sclerosis are of the Th1 subtype (Voskuhl et al 1993) while those infiltrating the conjunctiva in vernal conjunctivitis give Th2 clones (Maggi et al 1991). Lymphocyte populations from Graves ophthalmopathy (McLachlan et al 1994) and from Sjögren's syndrome, however, are neither entirely Th1 nor Th2 in phenotype. It would be valuable to determine completely the cytokine profile of T-helper lymphocytes in the cornea in CSK to determine whether the  $\gamma$ -IFN secreting cells reported here are

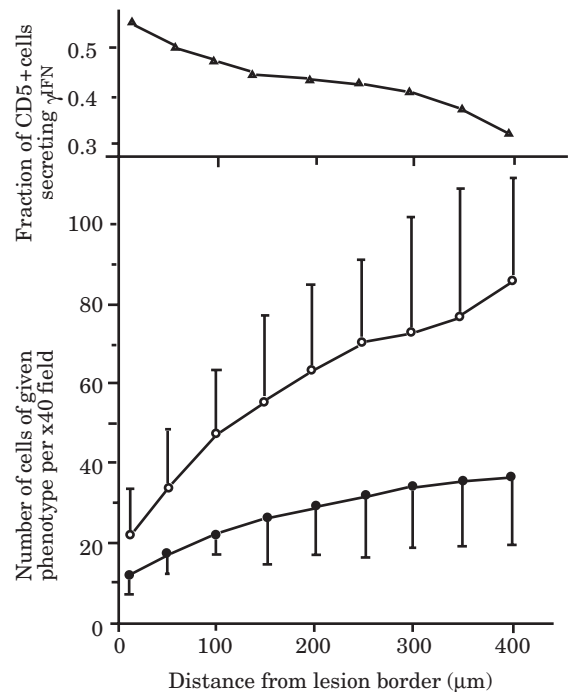


FIG 6: The mean number and SEM of CD5+ lymphocytes and  $\gamma$ IFN-secreting lymphocytes in 10 high power fields across the chronic superficial keratitis lesion in five dogs. —○— CD5+ lymphocytes; —●—  $\gamma$ IFN-secreting lymphocytes.

indeed a Th1 population, further indicating an autoimmune aetiopathogenesis for CSK.

This study has demonstrated that CD4-expressing lymphocytes are the predominant cell population infiltrating the corneal stroma in CSK. Similarities with cell types involved in diseases with defined autoimmune status suggests an autoimmune aetiology for CSK as does investigation of major histocompatibility class II antigen expression in the lesion (Williams 1997). This study provides preliminary data demonstrating that the cells expressing CD4 observed in the

CSK lesion are a mixed helper T-lymphocyte population: some cells produce IFN- $\gamma$  while others do not contain this cytokine. The lymphocytic, cytokine-producing phenotype of cells in the CSK lesion would naturally lead to the suggestion that cyclosporine A would be a useful therapeutic agent in the disease, as has been demonstrated (Williams et al 1995). The work presented in this study forms the basis for further investigation into the aetiology and pathogenesis of the disease.

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