

Immunopathological findings in conjunctival cells using immunofluorescence staining of impression cytology specimens

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Abstract

The conventional technique of impression cytology provides a non-invasive method for the evaluation of conjunctival epithelium alterations. Using indirect immunofluorescence procedures two inflammatory markers, class II MHC antigens HLA DR and the receptor to IgE (CD23), were sought in impression cytology specimens obtained from 80 patients. In normal subjects conjunctival epithelial cells did not show any reactivity. Only scattered dendritic cells were found to express class II antigens but not the receptor to IgE. In contrast patients with chronic conjunctivitis of various aetiologies, mainly infectious or allergic, had 40-100% of brightly positive conjunctival cells for one or both antigens. In these cases epithelial cells and goblet cells reacted similarly. Twenty four eyes in 12 patients with idiopathic dry eye syndrome disclosed results similar to those from normal conjunctival specimens. However 18 other specimens from patients suffering from idiopathic tear deficiency but undergoing multiple substitutive treatments for dry eye had moderate to strong positivity for HLA DR and/or the receptor to IgE (20-100% of cells). As these results were independent of the degree of squamous metaplasia the expression of these membrane markers may reflect local inflammation in addition to tear deficiency, possibly due to sensitisation to the eye drops used. These immunocytological techniques thus provide useful methods of investigating conjunctival inflammation and allergy. They may constitute valuable aid in the diagnosis and appropriate treatment of ocular surface disorders.

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Impression cytology is a quick and easy method of obtaining repeated and wide collections of superficial cells from the bulbar conjunctiva.¹⁻³ It allows quantitative assessment of the number of goblet cells in conjunctiva and a qualitative study of epithelial damage in various conjunctival diseases. This method has been mostly used in dry eye syndrome, a common pathological state in which the non-keratinised stratified conjunctival epithelium progressively loses goblet cells and differentiates into a non-secretory keratinised epithelium.²

Although impression cytology provides valuable information concerning dryness-related conjunctival disease, in most cases this method cannot point to the eventual involvement of inflammatory phenomena, isolated or associated

with tear deficiency. The eventual functional changes occurring in conjunctival epithelium, especially when cell morphology is not impaired, can only be assessed by immunocytological procedures using monoclonal antibodies to investigate the expression of various cell surface markers. Until now such procedures could not be routinely performed in standard impression cytology specimens, because cell collection is usually made on opaque cellulose membranes which must be chemically cleared before cytological examination. Routine cytological staining can be performed in such a way but not immunocytochemistry, which involves weak antigen-antibody complex formation. Following preliminary reports^{4,5} we routinely developed new impression cytology procedures which provided valuable specimens for immunocytochemistry, permitting examination of cell surface changes in conjunctival diseases.

As shown in previous immunocytological studies,⁶ class II histocompatibility antigens can be abnormally expressed by a variety of ocular cells in different pathological circumstances. Furthermore, as IgE are known to be directly involved in allergic phenomena and atopic keratoconjunctivitis,⁷ an immunocytological study was done using impression cytology specimens of the conjunctiva to investigate the expression of these two inflammatory markers in normal and diseased conjunctiva.

Material and methods

After obtaining informed consent ocular examinations were performed on 80 patients (160 eyes), aged 20 to 85 years. There were 17 randomly selected normal patients, 24 with dry eye syndrome, and 39 with conjunctivitis (of whom seven were affected in only one eye). Diagnosis of dry eye was made on the basis of subjective symptoms, the results of Schirmer test (less than 5 mm for 5 minutes), and tear break up time (less than 10 seconds). Subjective symptoms ranged from mild to severely disabling foreign body sensation or burning. Of these 24 patients only two had the diagnosis of Sjogren's syndrome, on the basis of association with dry mouth and serum autoantibodies (rheumatoid factor or antinuclear antibodies), as described by Pflugfelder *et al.*⁸ Another had Stevens-Johnson syndrome, responsible for severe ocular dryness. Although serum antibodies were not obtained in every patient with tear deficiency, the other patients with dry eyes had no clinical or biological sign evocative of systemic disease or local inflammatory impairment.

In the other 39 cases conjunctival specimens

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were obtained from patients with uni- or bilateral chronic conjunctivitis present for more than 2 weeks before examination. Although in some patients the origin of conjunctivitis was known (ocular allergy, bacterial or viral infection, topical medication, or contact lenses), in about 50% no accurate diagnosis could be made.

Impression cytology was performed using two different techniques. The first one was done in 10 normal patients and five with tear deficiency, following the previously described method of Iwata and Burris.⁴ For this procedure, Millicel-CM transparent Biopore membranes (PICM03050, Millipore, Bedford, MA) were cut into about 1 cm² pieces and pressed on the superior and temporal bulbar conjunctiva, in an area located at 2–8 mm from the limbus. The membranes were applied on the conjunctiva after topical anaesthesia and held there for a few seconds. Cytological and immunocytochemical procedures were then performed directly on the membranes, their transparency allowing microscopic examination. However, as Biopore membranes appeared to be difficult to use routinely because of their thinness and tendency to roll up during manipulations, we investigated a modified impression cytology technique with 0.20 µm cellulose acetate filters (Gelman, Apoteknia), as used in standard impression cytology collection,^{1,2} cut into approximately 4×8 mm sheets and placed on the bulbar conjunctiva. In the modified procedure, filters with the detached epithelial cells were firmly applied on gelatin coated slides, and immediately removed, permitting direct cytological and immunocytological examination on the slides. For this technique, it was found that the best cellular collection on glass slides could be obtained when cellulose acetate membranes were slightly wet. As this technique appeared to be more convenient than the Biopore procedure and provided good cytological results, it was adopted for all the other patients of this study. With both procedures, three specimens were collected in each eye (six for each patient), in three closely located but different areas of the superior and temporal bulbar conjunctiva.

Cytological staining was done using 5% cresyl violet in 0.9% NaCl for 30 minutes before rinsing with tap water and microscopic examination. By this method nuclei were stained in violet purple and mucins in pink. Indirect immunofluorescence procedures were performed on the other two specimens, according to previously described methods,⁶ using two monoclonal antibodies directed against the monomorphic region of class II histocompatibility antigens HLA DR (OKDR, Ortho Diagnostic System) and a membrane receptor for IgE, CD23 (B6, Coulter Clone).

Primary monoclonal antibodies, in a 1:50 dilution, were incubated for 1 hour, either directly on Biopore membranes or onto glass slides with conjunctival cells. After washing them in phosphate buffered saline (PBS), specimens were incubated for 30 minutes with the secondary antibody, fluorescein isothiocyanate-labelled anti-mouse immunoglobulin antiserum (Dakopatts). They were washed again in PBS, counterstained with

propidium iodide and mounted in mounting medium (AF1 mounting medium, Citifluor Ltd) before examination. For microscope examination, Biopore membranes were placed on glass slides and similarly mounted in glycerol containing medium. Nucleus counterstain using propidium iodide was adopted to more easily visualise negative cells and permit a rapid and accurate evaluation of the percentage of positive cells. Percentages of reactive cells were determined by counting at least 200 cells in each specimen. Damaged cells were not considered.

Results

Cytological and immunofluorescence staining using Biopore membranes or cellulose acetate filters showed similar results. Slightly more homogeneous and continuous sheets of conjunctival cells were obtained with Biopore membranes, whereas acetate cellulose filters resulted in large but more often discontinuous and interspaced islets of cells, with more numerous scattered cells. Both types of membranes showed gaps free of cells between spots of variable sizes. Application of membrane to gelatin coated slides did not result in significant cell loss, especially where goblet cell density was concerned, as assessed by the cresyl violet staining procedure. Numerous folds, however, were usually seen when using the very thin Biopore membranes, often accounting for a poor quality of sample collection and cytological examination. Immunocytological patterns were identical with both techniques.

STANDARD CYTOLOGICAL STAINING

As expected, cytological staining of conjunctival specimens from normal patients did not show any morphological anomaly (Fig 1). In contrast, specimens from patients with dry eye syndrome showed various degrees of morphological changes known as squamous metaplasia,² including a decrease in goblet cell density (found in all specimens) and more occasionally, a total loss of goblet cells, a flattening of cytoplasm, pyknotic change of nucleus, and even complete keratinisation of conjunctival epithelium. In specimens from patients with chronic conjunctivitis, more heterogeneous results were observed, from normal features to epithelial alterations close to those described above, such

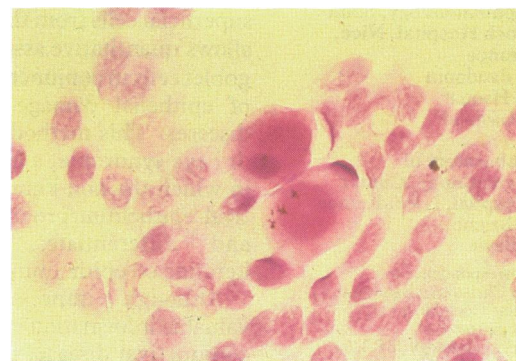


Figure 1 Two goblet cells in an impression cytology specimen from normal conjunctiva (cresyl violet, ×330).

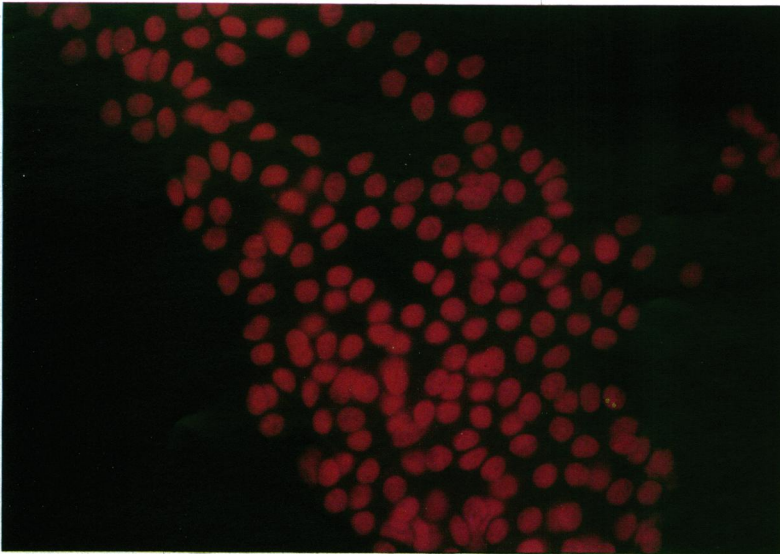


Figure 2 Negative immunofluorescence staining of an impression cytology specimen from a normal conjunctiva with anti-HLA DR monoclonal antibodies. Nuclei are counterstained with propidium iodide and appear in red. Cell membranes do not express HLA DR antigens and cannot be seen by epi-illumination (indirect immunofluorescence procedure, $\times 340$).

as decrease in goblet cell number or changes in nucleus cytoplasm ratios.

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Normal patients

The immunocytological study for class II antigen HLA DR and IgE receptor CD 23 remained negative in all normal conjunctival specimens (Fig 2) except for rare and isolated dendritic cells which strongly reacted with anti-HLA DR monoclonal antibody but not with anti-CD 23 monoclonal antibody. In some specimens, a very weak reactivity was seen on a minority of cells (less than 10%) with either or both antibodies.

Chronic conjunctivitis

The large majority of eyes with conjunctivitis (65 of 71), in contrast, strongly reacted with both monoclonal antibodies (Figs 3 and 4) which were expressed by 40–100% of epithelial cells of the conjunctiva (Table 1). When visible, goblet cells also expressed both markers. In most specimens, both antibodies reacted, but HLA DR positive cells were more numerous and more often found.

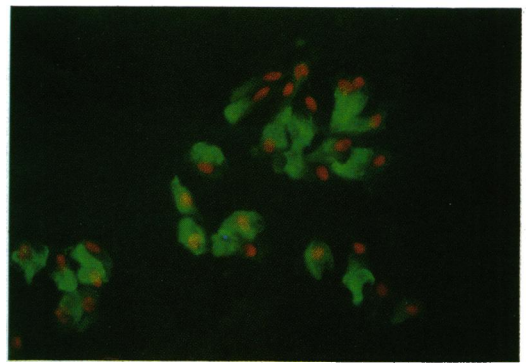


Figure 4 Similar green stained reactivity of conjunctival cells with anti-CD23 monoclonal antibodies in a patient with bilateral chronic conjunctivitis (indirect immunofluorescence, propidium iodide counterstain, $\times 220$).

In cases of unilateral conjunctivitis, a striking difference was seen between both eyes, the normal one remaining negative, while the pathological one strongly reacted. These results were independent of the clinical history, the origin of the conjunctival disease when known, or the degree of cytological anomalies. Conjunctival cells, however, remained negative for both markers in three patients (six eyes) complaining of chronic ocular burning and redness, but without any objective sign of ocular dryness and considered as having chronic conjunctivitis. Two of them had received steroid treatment prior to specimen collection, which could have inhibited the expression of inflammatory markers by conjunctival cells. It was noteworthy that none of the positive specimens were obtained from patients receiving topical steroids.

Patients with tear deficiency

For dry eye syndrome various results were observed. Twenty four specimens with clinical dryness and cytological features of squamous metaplasia remained totally negative for both antigens. Two patients with the diagnosis of Sjogren's syndrome were positive for HLA DR antigens (90–100% of conjunctival cells on both eyes), but not for IgE receptors (less than 10%). The patient with Stevens-Johnson syndrome was strongly positive for both antibodies (95% of positive cells). The 18 other conjunctival specimens from patients with clinical aqueous tear deficiency showed positive but often weaker reactivity (Fig 5) for both monoclonal antibodies (Table 1). Again, the pattern of reactivity and the percentages of positive cells were not related to clinical and cytological features and remained independent of the degree of squamous metaplasia observed in conjunctival cells. Cytological examination did not show a significant difference between negative and positive specimens, as some specimens with total goblet cell loss were negative, whereas very mild forms of squamous metaplasia could be strongly positive. In most cases, percentages of positive cells were similar for both antibodies, but in four eyes the proportion of CD 23 positive cells was higher (60 to 70%) than seen for HLA DR (20–30%). It is noteworthy that, except for two patients (four eyes), all those with positive conjunctival cells had a long term course of topical medication for

Figure 3 HLA DR antigen expression by a large majority of conjunctival cells in a case of chronic conjunctivitis. Positive immunostaining appears in green, whereas nuclei are yellow-orange by superimpression of green immunoreactivity of cell membranes and red staining of counterstained nuclei (indirect immunofluorescence, propidium iodide counterstain, $\times 240$).

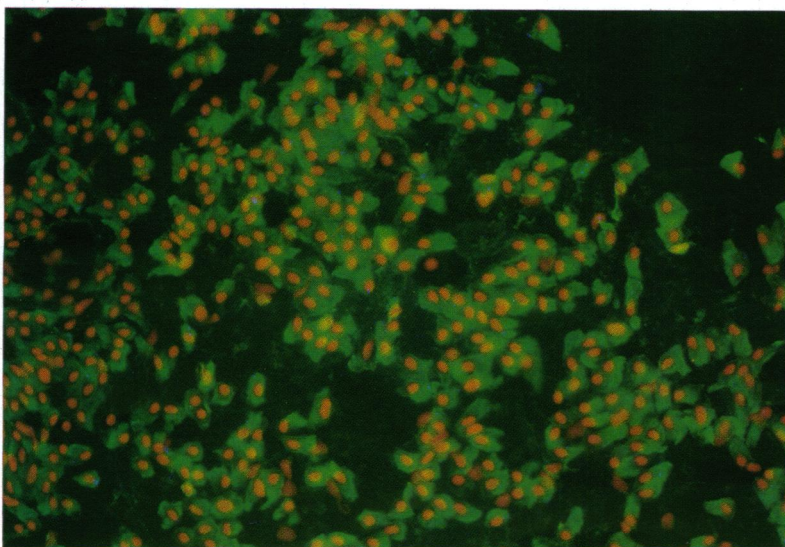


Table 1 Immunostaining reactivity of impression cytology specimens from normal conjunctiva and in ocular surface disorders. Mean percentages of reactivity were calculated for positive specimens only

Clinical diagnosis	No of specimens	Mean percentages of cells (SD)		Range (%)		No of positive specimens	
		HLA DR	CD 23	HLA DR	CD 23	HLA DR	CD 23
Normal	(41)*	2 (3.6)	2.5 (4.3)	0-10	0-10	7	5
Conjunctivitis	(71)	79 (22)	54 (29)	40-100	40-100	65	58
Dry eye	(48)	59 (27)	60 (19)	20-100	30-100	24	18

*17 normal patients and seven contralateral eyes in unilateral conjunctivitis

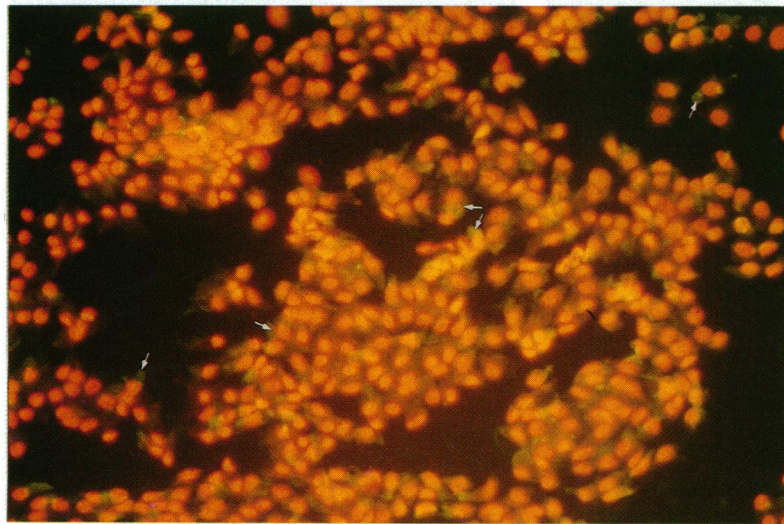
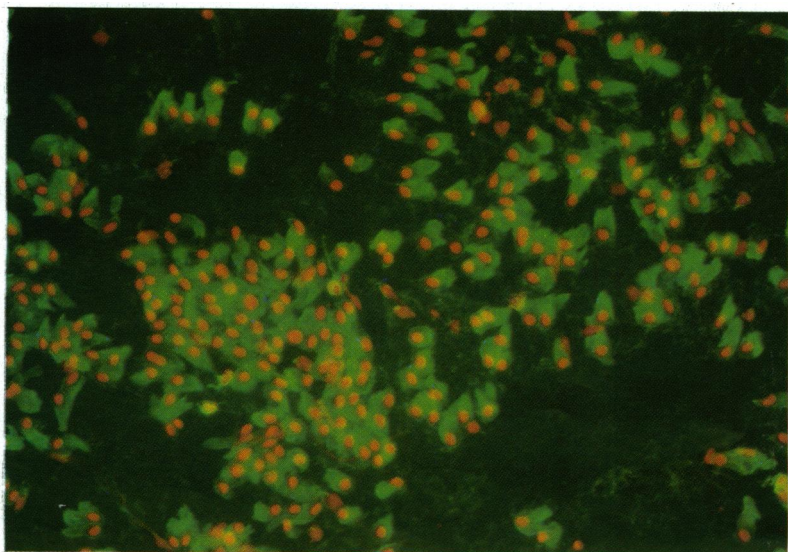


Figure 5 Weaker immunofluorescence reactivity of anti-HLA DR antibodies in some conjunctival cells from a patient with dry eyes. Comparing with Figure 3 or Figure 6, the green immunostaining (arrows) is weak and can be seen only in a minority of epithelial cells. Nuclei are counterstained in orange by propidium iodide ($\times 200$).

Figure 6 In contrast to Figure 5, conjunctival cells from another patient with dry eyes but showing clinical intolerance to topical medications, strongly express HLA DR antigens on their membrane (green immunofluorescence staining, orange nucleus counterstain, $\times 200$).



dry eye syndrome, and that at least two had developed intolerance to their drugs (Fig 6).

Discussion

The technique of impression cytology was first described by Egbert *et al*¹ and provided a simple procedure for cytological assessment of morphological changes occurring in conjunctival cells. These anomalies, designed as squamous metaplasia have been observed in various types of diseases including atopic keratoconjunctivitis, keratoconjunctivitis sicca, xerophthalmia, ocular pemphigoid, Stevens-Johnson disease, or chemical burns.² Most of the cytological studies done in conjunctival impression cytology specimens consisted of counting goblet cell

density and determining cellular characteristics, such as morphological changes of the nucleus, nucleus cytoplasm ratios, metachromic changes of cytoplasmic colour, and emergence of keratinisation.^{2,3} A classification system defining six stages in squamous metaplasia² provided an additional application for impression cytology by allowing estimation of the severity of the conjunctival disease and an evaluation of progression or improvement of conjunctival changes.

In addition to morphological analysis, the use of modifications in conventional impression cytology procedure to perform immunocytological studies was thus of interest, by permitting investigation of the expression of various cell markers in normal and diseased conjunctiva. Transferring conjunctival cells from cellulose filters onto gelatin coated slides was a simple way of obtaining a large number of cells on transparent holders, avoiding fixative or chemical clearing agents for filter strips that would not be compatible with immunocytochemistry. We found this procedure to be more convenient than the use of transparent Biopore membranes, first described by Iwata and Burris,⁴ and more recently used by Pflugfelder *et al*,⁵ because of the excessive thinness of these membranes, which frequently roll up during specimen collection and immunostaining procedures resulting in cell damage and poorer quality of conjunctival cell sheets. Cytological examination of specimens after transfer on glass slides showed a good collection of epithelial and goblet cells, permitting good quality cytological and immunochemical examination. Although both methods provided interesting results, we adopted the transfer procedure for routine collection and standard immunocytochemistry.

Class II histocompatibility antigens are membrane glycoproteins which are normally restricted to cells of the immune system, such as macrophages, B lymphocytes, and activated T cells, on which they play a major role in the initiation of the immune response to an antigen. Their deviant expression has been observed on various types of epithelial or mesenchymal cells involved in autoimmune diseases,^{9,10} and it has been suggested that the aberrant expression of HLA DR by the target cells could play a crucial role in the activation of the immune system and development of inflammatory phenomena.¹¹ In previous immunocytological studies performed in intraocular proliferative diseases, we found class II antigen expression at the surface of different types of cells, such as vascular endothelial cells in proliferative diabetic retinopathy and retinal or ciliary pigment epithelial cells in proliferative vitreoretinopathy.⁶

Histological examinations of biopsy or autopsy specimens have shown that normal conjunctiva does not express class II antigens.^{12,13} Class II antigen expression by conjunctival epithelial cells has been previously found using impression cytology procedures in patients with two autoimmune ocular surface disorders, Stevens-Johnson, and Sjogren's syndrome.¹⁴ In another study, swabs for detection of class II expression by conjunctival cells had been taken from patients with trachoma and demonstrated a strong expression associated with active trachoma,¹⁵ but

until now impression cytology specimens had not been used routinely for the immunocytological investigation of class II antigen expression in ocular surface disorders. The present study confirms the histological assessment that conjunctival epithelium does not normally express HLA DR antigens, except in rare scattered class II positive dendritic cells, which act as antigen-presenting cells to the immune system. We also demonstrated that conjunctival epithelial cells can express class II antigens in various inflammatory conditions clinically defined as chronic conjunctivitis, in which class II antigen expression probably represents more of an epiphenomenon related to local inflammation, than a causal involvement of the conjunctiva.

More interestingly, patients with dry eye syndrome showed different immunocytological features. The immunoreactivity patterns were not related to cytological results and degree of squamous metaplasia. In 24 eyes with dry eye syndrome, conjunctival epithelial cells disclosed immunoreactivity similar to that observed in normal patients. Six eyes of patients with Stevens-Johnson or Sjogren's syndrome and 18 other specimens from patients with idiopathic dry eye syndrome, however, showed an abnormal expression of class II antigens. These patients had clinical diagnosis of dry eye syndrome and various degrees of squamous metaplasia on cytological examination. As HLA DR expression was independent of the stage of conjunctival keratinisation, it may be hypothesised that conjunctival cells were not induced to express class II antigens by ocular dryness itself, but that inflammatory phenomena were associated, possibly resulting from concomitant infection, allergy, toxic effects of topical medications, or direct autoimmune involvement of conjunctiva in Stevens-Johnson or Sjogren's syndrome. Mechanisms of expression of class II antigens are not well known, but interferon- γ has been shown to be a major inducer of such expression by the target cells, both in vivo, in animal models,¹⁶ and in vitro, on a large variety of ocular cells.^{17,18}

Similar results were observed with another inflammatory marker, CD 23, which acts as low affinity receptor to IgE, an immunoglobulin which mediates type I hypersensitivity and is increased in serum and tear samples from patients with atopic keratoconjunctivitis.⁶ This cell surface antigen has been mainly studied in cells of the immune system on which its expression is enhanced by mediators of inflammation, such as platelet activating factor.¹⁹ Platelet activating factor is a potent mediator that induces platelet aggregation and activation of the immune system, and creates conjunctival inflammation when topically administered to rabbits or humans.²⁰

The presence of this receptor at the conjunctival level, as well as HLA DR expression, in some patients with clinical dry eye syndrome, may therefore be related to an associated topical inflammation, that should be treated concomitantly, possibly with anti-inflammatory agents and by avoiding additional sensitisation with inappropriate substitutive medications for tear deficiency. It was not

possible in this preliminary report to assess more precisely the consequences of steroid treatments on class II antigen expression by conjunctival epithelium, but it was noteworthy that two of the only three patients with chronic conjunctivitis who exhibited negative reactivity had received topical steroids prior to specimen collection.

Although additional studies need to be performed to evaluate the effect of specific therapeutic agents for topical inflammation, the use of immunocytological procedures in addition to the conventional impression cytology technique may now offer valuable information in conjunctival diseases. Routine immunocytology may be helpful in evaluation and treatment of chronic ocular surface disorders, especially dry eye syndrome, when clinical and current laboratory criteria do not provide the accurate diagnosis and when treatment cannot lead to significant relief.

- 1 Egbert PR, Laube S, Maurice DM. A simple conjunctival biopsy. *Am J Ophthalmol* 1977; 84: 798-801.
- 2 Tseng SCG. Staging of conjunctival squamous metaplasia by impression cytology. *Ophthalmology* 1985; 92: 728-33.
- 3 Rolando M, Terragna F, Giordano G, Calabria G. Conjunctival surface damage distribution in keratoconjunctivitis sicca. An impression cytology study. *Ophthalmology* 1990; 200: 170-6.
- 4 Iwata RT, Burris TE. Impression cytology immunocytochemistry techniques. *Invest Ophthalmol Vis Sci* 1989; 30 (suppl): 521.
- 5 Pflugfelder SC, Huang AJW, Feuer W, Chuchovski PT, Pereira IC, Tseng SCG. Conjunctival cytologic features of primary Sjogren's syndrome. *Ophthalmology* 1990; 97: 985-95.
- 6 Baudouin Ch, Fredj-Reygrobellet D, Gordon WC, et al. Immunohistologic study of epiretinal membranes in proliferative vitreoretinopathy. *Am J Ophthalmol* 1990; 110: 593-8.
- 7 Tuft SJ, Kemeny DM, Dart JKG, Buckley RJ. Clinical features of atopic keratoconjunctivitis. *Ophthalmology* 1991; 98: 150-8.
- 8 Pflugfelder SC, Tseng SCG, Pepose JS, Fletcher MA, Klimas N, Feuer W. Epstein-Barr virus infection and immunologic dysfunction in patients with aqueous tear deficiency. *Ophthalmology* 1990; 97: 313-23.
- 9 Hanafusa T, Pujol-Borrell R, Chiovato L, Russell RCG, Doniach D, Bottazzo GF. Aberrant expression of HLA DR antigen on thyrocytes of Graves' disease: relevance for autoimmunity. *Lancet* 1983; ii: 1111-5.
- 10 Foulis AK, Farquharson MA. Aberrant expression of HLA DR antigens by insulin containing cells in recent onset type I diabetes mellitus. *Diabetes* 1986; 35: 1215-25.
- 11 Bottazzo GF, Pujol-Borrell R, Hanafusa T, Feldmann M. Role of aberrant HLA DR expression and antigen presentation in induction of endocrine autoimmunity. *Lancet* 1983; ii: 1115-8.
- 12 Rodrigues MM, Rowden G, Hackett J, Bakos I. Langerhans cells in normal conjunctiva and peripheral cornea of selected species. *Invest Ophthalmol Vis Sci* 1981; 21: 759-65.
- 13 Baudouin Ch, Fredj-Reygrobellet D, Gastaud P, Lapalus Ph. HLA DR and DQ distribution in normal human ocular structures. *Curr Eye Res* 1988; 7: 903-11.
- 14 Blodi BA, Pflugfelder SC, Crouse C, Levine J, Atherton SS. Immunocytological evaluation of class II MHC antigen expression on the conjunctival epithelium. *Invest Ophthalmol Vis Sci* 1990; 31 (suppl): 62.
- 15 Mabey DCW, Bailey RL, Dunn D, et al. Expression of MHC class II antigens by conjunctival epithelial cells in trachoma: implications concerning the pathogenesis of blinding disease. *J Clin Pathol* 1991; 44: 285-9.
- 16 Hamel Ch, Detrick B, Hooks JJ. Evaluation of Ia expression in rat ocular tissues following inoculation with interferon gamma. *Exp Eye Res* 1990; 50: 173-82.
- 17 Foets BJJ, Van Den Oord JJ, Biliau A, Van Damme JV, Missotten L. Heterogeneous induction of major histocompatibility complex class II antigens on corneal endothelium by interferon gamma. *Invest Ophthalmol Vis Sci* 1991; 32: 341-5.
- 18 Baudouin Ch, Fredj-Reygrobellet D, Jambou D, Gastaud P, Lapalus Ph. HLA DR and DQ expression on human retinal pigment epithelial cells in vitro. *Graefes Arch Clin Exp Ophthalmol* 1990; 228: 86-9.
- 19 Wardlaw AJ, Moqbel R, Kurihara K, Walsh G, Kay B. Role of PAF-acether in leucocyte activation and chemotaxis. In: Braquet, P ed. *The role of platelet activating factor in immune disorders*. Basel: Karger, 1988, vol 2, 1-9.
- 20 George MA, Smith LM, Berdy GJ, Abelson MB. Platelet activating factor induced inflammation following topical ocular challenge. *Invest Ophthalmol Vis Sci* 1990; 31 (suppl): 63.