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Immunohistochemical study of human pterygium

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Summary. The purpose of this study has been to evaluate the immunohistochemical characteristics of human pterygial tissues in order to ascertain the possible contribution of an immunological mechanism in the pathogenesis of pterygium and to investigate the presence in the pterygial tissues of some melanoma-associated antigens, in order to evaluate if there may be a small possibility of correlation of the two diseases.

Human biopsy specimens of pterygium were obtained by surgery for pterygium excision. Tissue segments were fixed and processed for paraffin embedding. Microtome sections were treated for the immunohistochemical demonstration of IgA, IgM, IgG, CD3, CD20, CD68, HLA-DR, Protein S100, HMB45, and Melan A using the avidin-biotin peroxidase method or the streptavidin biotin-alkaline phosphatase method.

The findings suggest that all the effector components of the mucosal immune system are present in the human pterygium and, among the most sensitive markers for melanoma, only S100 shows immunoreactivity.

An immunopathogenetic mechanism seems to be responsible for the pathogenesis of pterygium, perhaps being caused by pre-existing conjunctivitis or microtrauma in combination with the patient's predisposition. No correlation between pterygium and melanoma was found.

Key words: Immunohistochemistry, Immune system, Pterygium, Human

Introduction

Pterygium, a disease of unknown origin and pathogenesis, is a chronic condition characterized by the encroachment of a fleshy, triangular portion of the bulbar conjunctiva into the cornea (Peckar, 1972; Vaughan and Ashbury, 1977). Pterygium consists of an epithelium of the conjunctival type that covers a highly vascularized structure of blood and loose fibrous connective tissue (Elliot, 1962). It is more common on the nasal side of the eye and is often bilateral. There is some evidence that ultraviolet light has a major impact on the pathogenesis of pterygium (Moran and Hollows, 1984; Coroneo, 1993; Kwok and Coroneo, 1994). In fact, irritation of the eye by ultraviolet radiation in sunny, dry, dusty areas and repeated microtrauma can lead to the development of pterygium in susceptible individuals. Histologically, this lesion shows alternately thickening or thinning of the epithelium, with elastoid and basophilic degeneration of the underlying connective tissues (Duke Elder, 1974).

The aetiology and pathogenesis of pterygium are not clearly understood; some investigators have suggested an inflammatory process (Raizada and Bathnagar, 1976; Wong, 1978), whereas others have noted a primary degeneration of the cornea, followed by fibroblastic proliferation (Elliot, 1966; Cilova-Atanasova, 1971). Austin et al. (1983) suggested that elastodysplasia, followed by elastodystrophy, may be involved in the process of pterygium formation. Other investigators believe that mast cells are actively involved in the genesis and progress of pterygium (Butrus et al., 1995; Nakagami, 1999). Various other theories, including anatomic (Lin and Huang, 1954), neoplastic (Hilgers, 1960), sociological (Qi, 1966), and allergic factors (Pinkerton et al., 1984) have been proposed. Polymerase chain reaction (PCR) studies have revealed viral presence (herpes simplex virus, cytomegalovirus, human papillomavirus) in pterygia (Varinli et al., 1994; Spandidos et al., 1997; Detorakis et al., 2000). A familial predisposition for pterygium was first observed in 1893 (Zhang, 1987), and recent studies demonstrate that pterygia often display the phenomenon of loss of heterozygosity (LOH), suggesting that tumour suppressor genes could be involved in the development of this lesion (Detorakis et al., 1998). Some investigators have suggested that an immunological mechanism, possibly type I hypersensitivity, may contribute to the pathogenesis of pterygium (Pinkerton et al., 1984; Liu and Yang, 1993).

Immunopathogenetic mechanisms (Ioachim-Velogianni et al., 1995) and overexpression of the extracellular matrix (Karukonda et al., 1995) have recently been discussed in relation to the pathogenesis of

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pterygium. Nevertheless, the pathogenesis of pterygium is still not completely clear so that we can better understand its management in order to prevent recurrence.

The purpose of this study has been to evaluate the immunohistochemical characteristics of human pterygial tissues in order to ascertain the possible contribution of an immunological mechanism in the pathogenesis of pterygium.

Moreover, since pterygia represent a precursor lesion of actinic keratosis, considered as a precancerous condition related to excessive sun exposure, U.V. irradiation being the mayor environmental predisposing factor in the pathogenesis of both pterygium and melanoma, and taking into account that the chromosomal region 9p21 displays high incidence of LOH in the pterygium (Detorakis et al., 1998) and melanoma (Fountain et al., 1992; Holland et al., 1994; Isshiki et al., 1994; Walker et al., 1994; Healy et al., 1996; Greene, 1999; Birindelli et al., 2000), we attempted to investigate the presence, in the pterygial tissues, of some melanoma-associated antigens, in order to evaluate if there may be a small possibility of correlation of the two diseases.

Materials and methods

Human biopsy specimens of pterygium were obtained by surgery from 48 patients (35 males and 13 females) ranging in age from 30 to 82 years at the time of surgery for pterygium excision. All patients had normal immunological responses.

Tissue segments were fixed by immersion in cold 10% paraformaldehyde in 0.2M phosphate buffer, pH 7.3, for 4-6 h, and processed for paraffin embedding. Microtome sections (6-7 µm) were treated for the immunohistochemical demonstration of sIgA, IgM and IgG using the ABC peroxidase method: they were rehydrated in PBS, pre-treated with 0.1% trypsin (Sigma, St Louis, MO) in PBS at 37 °C for 10-20 min to retrieve the antigens, then immersed for 30 min in a solution of 100% methyl alcohol and 30% perhydrol to inactivate endogenous peroxidase.

The sections were treated for 45 min with 10% normal goat serum (NGS) or normal horse serum (NHS) in PBS. Rabbit polyclonal antibody to human sIgA (Cappel, Durham, NC; 1:4000), mouse monoclonal antibody to human IgM (clone R1/69, Dako, Glostrup, Denmark; 1:100), and mouse monoclonal antibody to human IgG (clone A57H, Dako, Glostrup, Denmark; 1:100) were used as primary antisera. Both anti-human IgA and anti-human IgG were $F(ab^1)2$ fragments. Biotinylated anti-rabbit and anti-mouse IgG were used as secondary antisera (Vector Laboratories, Burlingame, CA, USA; 1:200) for 30 min at room temperature. The sections were further incubated in acetyl-avidin biotinylated-peroxidase complex (Biospa, Milano, Italy; 1:250) for 30 min at room temperature, reacted with 3,3'-diaminobenzidine (Sigma, St. Louis, MO, USA) and then counterstained with hematoxylin, dehydrated and mounted in Entellan (Merck, Frankfurter, Germany). The sections were thoroughly rinsed in PBS between each step.

Furthermore adjacent sections were incubated using a rabbit polyclonal anti-human IgA specific for alpha-chains, $F(ab^1)2$ fragment, (Dako, Glostrup, Denmark; working dilution 1:4000) as primary antiserum for the concurrent demonstration of IgA class immunoglobulins.

Other microtome sections were treated for the immunohistochemical demonstration of CD3, CD20, CD68, HLA-DR, Protein S100 and melanoma-associated antigens using the avidin-biotin peroxidase complex or the alkaline phosphatase streptavidin method. Antigen retrieval was performed by immersion in 0.1% trypsin solution in PBS at 37 °C for 5-10 min or by microwave heating for 5 min x 4 (total of 20 min) in 10 mM citrate buffer solution (pH 6.0) or 1 mM EDTA-NaOH buffer solution (pH 8.0). Mouse monoclonal antibody to human T-cells CD3 (clone PC3/188A, Dako, Glostrup, Denmark; 1:50), mouse monoclonal antibody to human B-cells CD20 (clone L26, Dako, Glostrup, Denmark; 1:500), mouse monoclonal antibody to human macrophages CD68 (clone PG-M1, Dako, Glostrup, Denmark; 1:100), mouse monoclonal LN3/HLA-DR to human HLA-DR antigen (clone LN3, Clonab®, Biotest, Milan, Italy, 1:4), rabbit polyclonal antibody to cow Protein S100 (Dako, Glostrup, Denmark; 1:100), and mouse monoclonal anti-human Merlan A (clone A103, Dako, Glostrup, Denmark; 1:100), were used as primary antisera and biotinylated anti-rabbit and anti-mouse IgG were used as secondary antisera. The sections subjected to the alkaline phosphatase streptavidin method were further incubated in alkaline phosphatase streptavidin (Vector Laboratories, Burlingame, CA, USA; 1:1000) for 30 min at room temperature, reacted with Fast Red Substrate System (Dako Glostrup, Denmark) or with Dako, Fuchsin+ Substrate-Chromogen (Dako, Glostrup, Denmark) and then counterstained with Mayer hematoxylin and mounted in glycerol gelatin (Sigma, St. Louis, MO, USA).

As control tissues for the melanoma-associated antigens, sections of non-diseased human conjunctiva and sections of human melanoma were employed as negative and positive control respectively.

In the control sections of pterygium the specificity of the antisera was tested by replacing the primary antibodies with normal serum.

Results

The fully developed pterygium was covered by conjunctival epithelium, stratified with flat cells on the surface of the head and the neck, but cylindrical in the numerous folds and furrows and at the base. The epithelium showed alternative areas of thinning or thickening. Beneath the epithelium, an area of elastosis and basophilic degeneration of the substantia propria collagen with increased vascularity was present (Fig. 1).

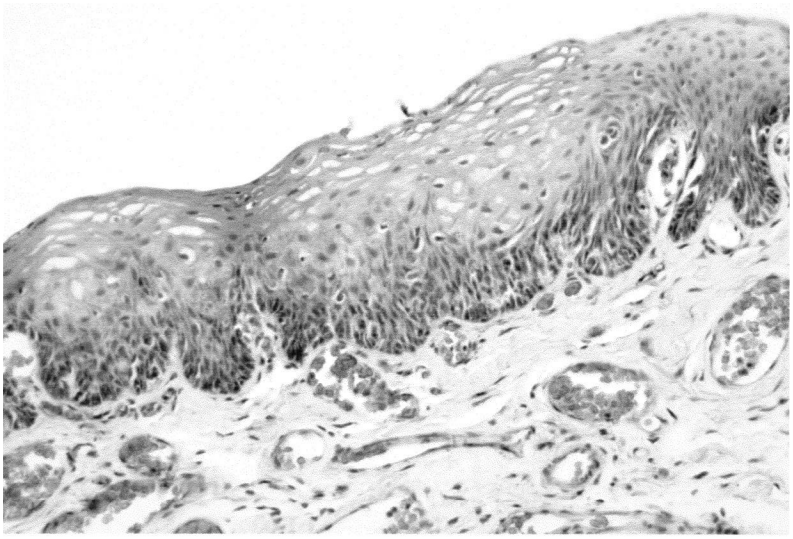

Fig. 1. Hematoxylin-eosin. The epithelium shows alternative areas of thinning or thickening, and an area of elastosis and basophilic degeneration of the substantia propria is present. x 200

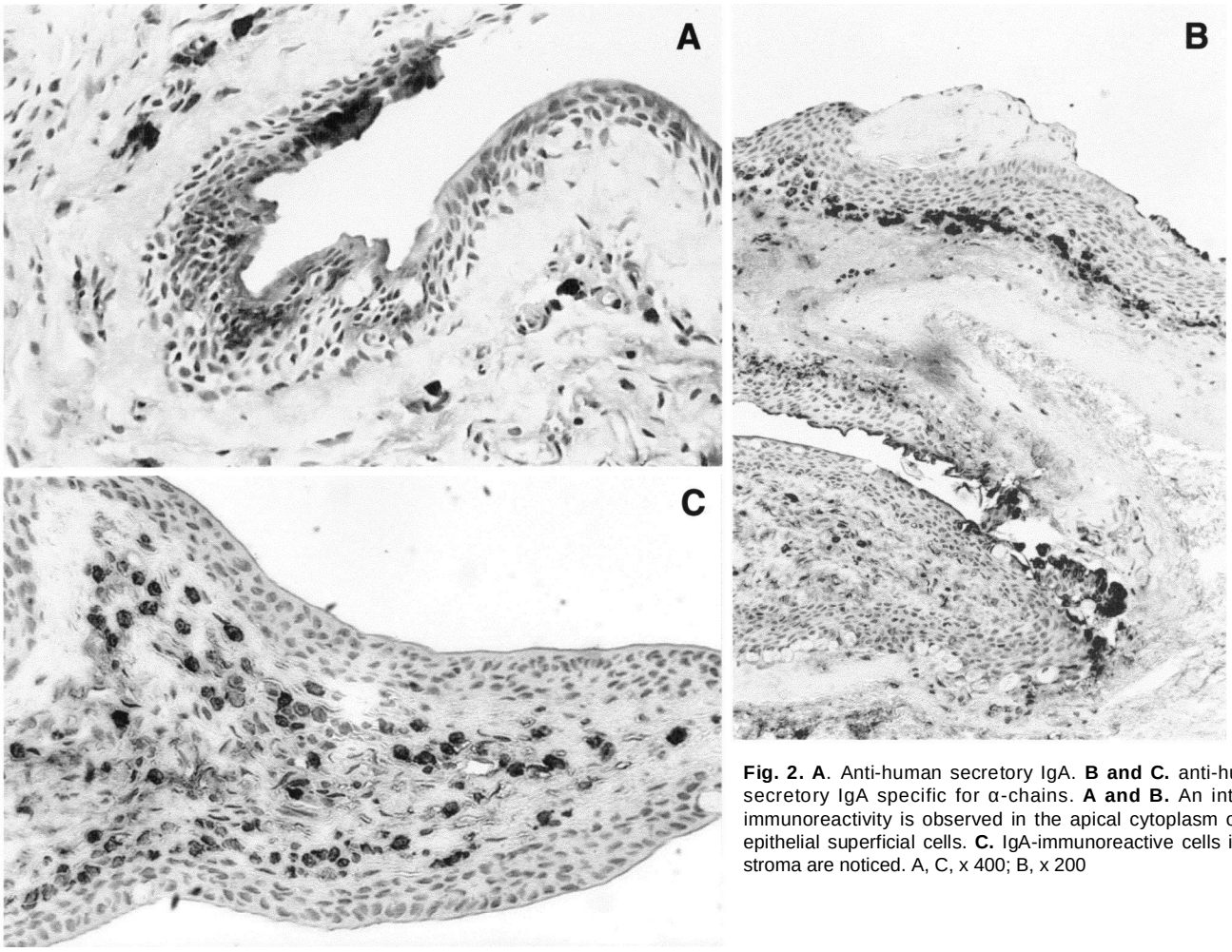

Fig. 2. A. Anti-human secretory IgA. **B and C.** anti-human secretory IgA specific for α-chains. **A and B.** An intense immunoreactivity is observed in the apical cytoplasm of the epithelial superficial cells. **C.** IgA-immunoreactive cells in the stroma are noticed. A, C, x 400; B, x 200

An intense immunoractivity for sIgA was observed in the apical cytoplasm of the epithelial superficial cells (Fig. 2A,B), and IgA-immunoreactive cells in the stroma were noticed (Fig. 2C). The staining for anti-human secretory IgA, specific for α-chains, also showed discontinuous areas of less dense or completely absent immunoreactivity (Fig. 2B,C), and a large amount of immunocompetent cells and deposition of immunoglobulins around the basement membrane of the epithelial layer was present (Fig. 2B).

An evident immunoreactivity for IgM and IgG was observed in the superficial layers of the epithelium (Fig. 3A,B), especially in the apical epithelial cells (Fig. 3A). The staining for IgM showed discontinuous areas of weak or completely absent immunoreaction (Fig. 3A). A large number of IgG-immunoreactive cells, recognized as plasma cells, were detected either in the subepithelial connective tissue or deeper in the stroma (Fig. 3B).T-lymphocytes, stained with anti-human CD3, were either scattered in the substantia propria or inside the epithelium, especially in the basal layers (Fig. 4A), while B-lymphocytes, stained with anti-human CD20, were occasionally detected in the lamina propria (Fig. 4B). Numerous macrophages, stained with anti-human CD68, were distributed throughout the lamina propria and subepithelially. CD68-positive cells were also observed inside the epithelium (Fig. 4C). The staining for HLA-DR antigen and S100 Protein was expressed in both the stroma and the epithelium. Numerous HLA-DR-immunoreactive cells with dendritic-like processes were observable in the epithelium. In the stroma, most of the cells expressing HLA-DR antigen were vascular endothelial cells (Fig. 5A). S100-immunoreaction was present mostly in the epithelium, particularly in the basal portion. Some of these immunoreactive cells with dendritic-like processes were considered to be Langerhans' cells. Numerous S100-positive cells were also detected in the substantia propria, either scattered near the epithelium or in groups around the blood vessels (Fig. 5B).

Among the most sensitive markers for melanoma, in the pterygium and in non-diseased conjunctiva only S100 showed immunoreactivity (Figs. 6A, 7A). In these tissues, no immunoreaction for HMB45 (Figs. 6B, 7B), and Melan A (Figs. 6C, 7C) in any area was observed. All the sections of human melanoma showed a positive immunostaining to the melanoma-associated antigens (Fig. 7D,E,F).

Immunostaining in the control sections treated with normal serum was completely abolished (Fig. 8A,B).

No sex- and age-related difference in the distribution pattern of the reactions was noticed.

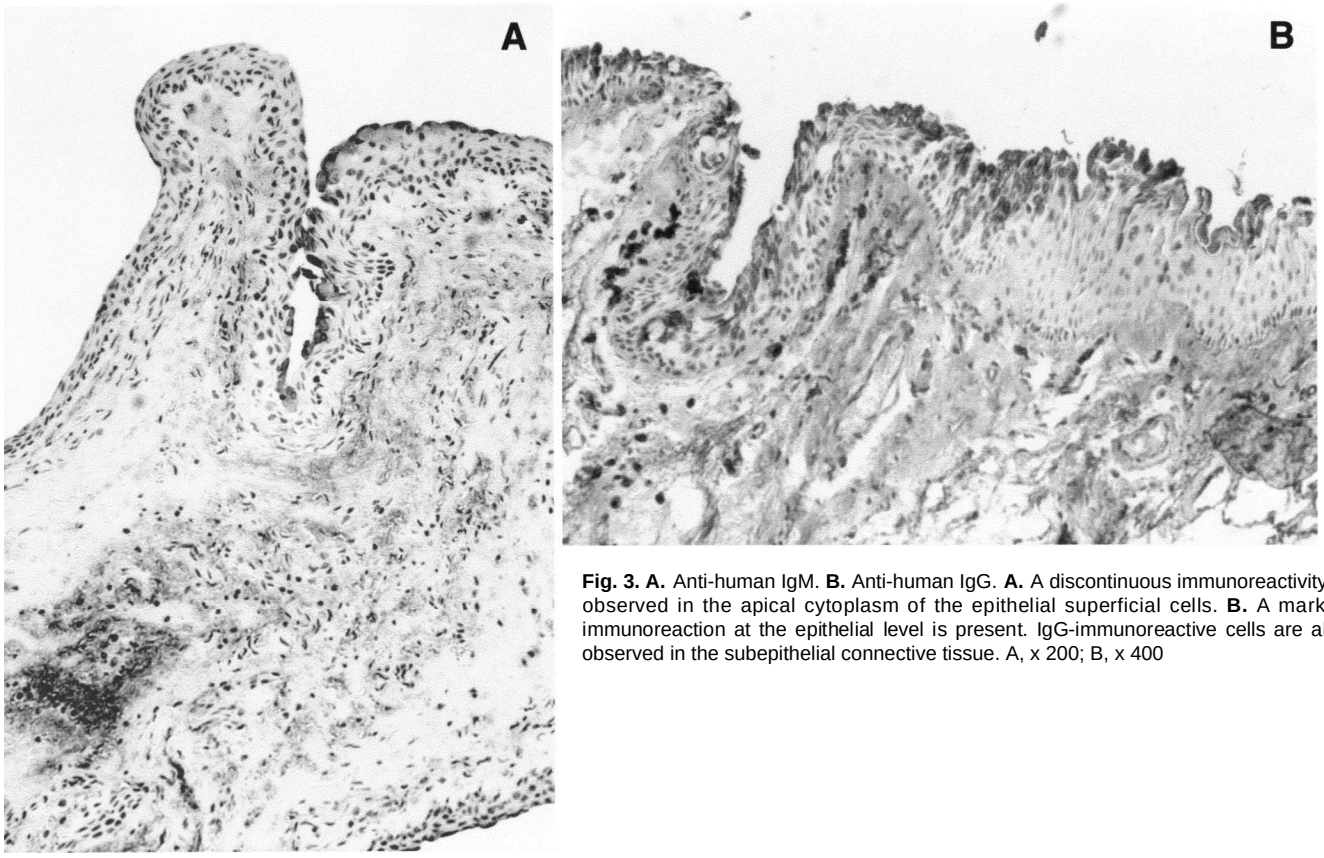

Fig. 3. A. Anti-human IgM. **B.** Anti-human IgG. **A.** A discontinuous immunoreactivity is observed in the apical cytoplasm of the epithelial superficial cells. **B.** A marked immunoreaction at the epithelial level is present. IgG-immunoreactive cells are also observed in the subepithelial connective tissue. A, x 200; B, x 400

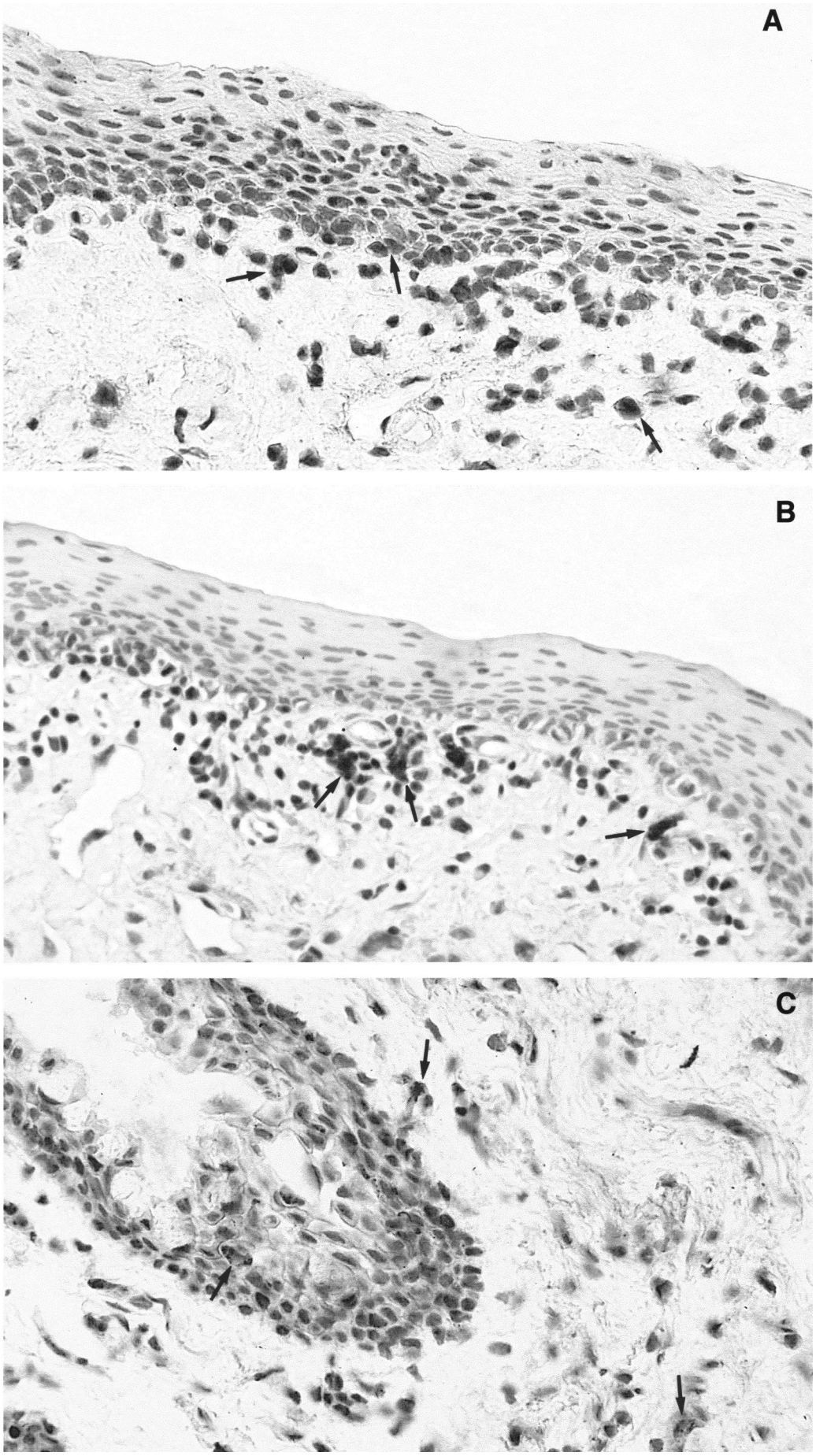

Fig. 4. A. Anti-human T-cells (CD3)**. B.** Anti-human B-cells (CD20). **C.** Anti-human macrophages (CD68).
A and B. Numerous T-cells and occasional B-cells are detected in the lamina propria (arrows). T-cells are also observed in the basal layer of the epithelium (arrows). **C.** Numerous CD68-positive cells are noticed (arrows). x 400

Discussion

A number of defense mechanisms protect the external eye against invasion by microorganisms. The structural integrity and blinking action of the eyelids, the intact epithelium of the conjunctiva and cornea, the tears with their specific and non-specific antimicrobial substances, and the conjunctival bacteria are all important for outer eye defense. In addition, the conjunctiva and the lacrimal gland participate in the common mucosal immune system. In fact, the local immunity of the ocular surface is governed by conjunctiva-associated lymphoid tissue (CALT), which is considered to be an integral part of the mucosal immune system, secretory IgA and immunocytes (Shoji et al., 1998) Immunoglobulins, particularly

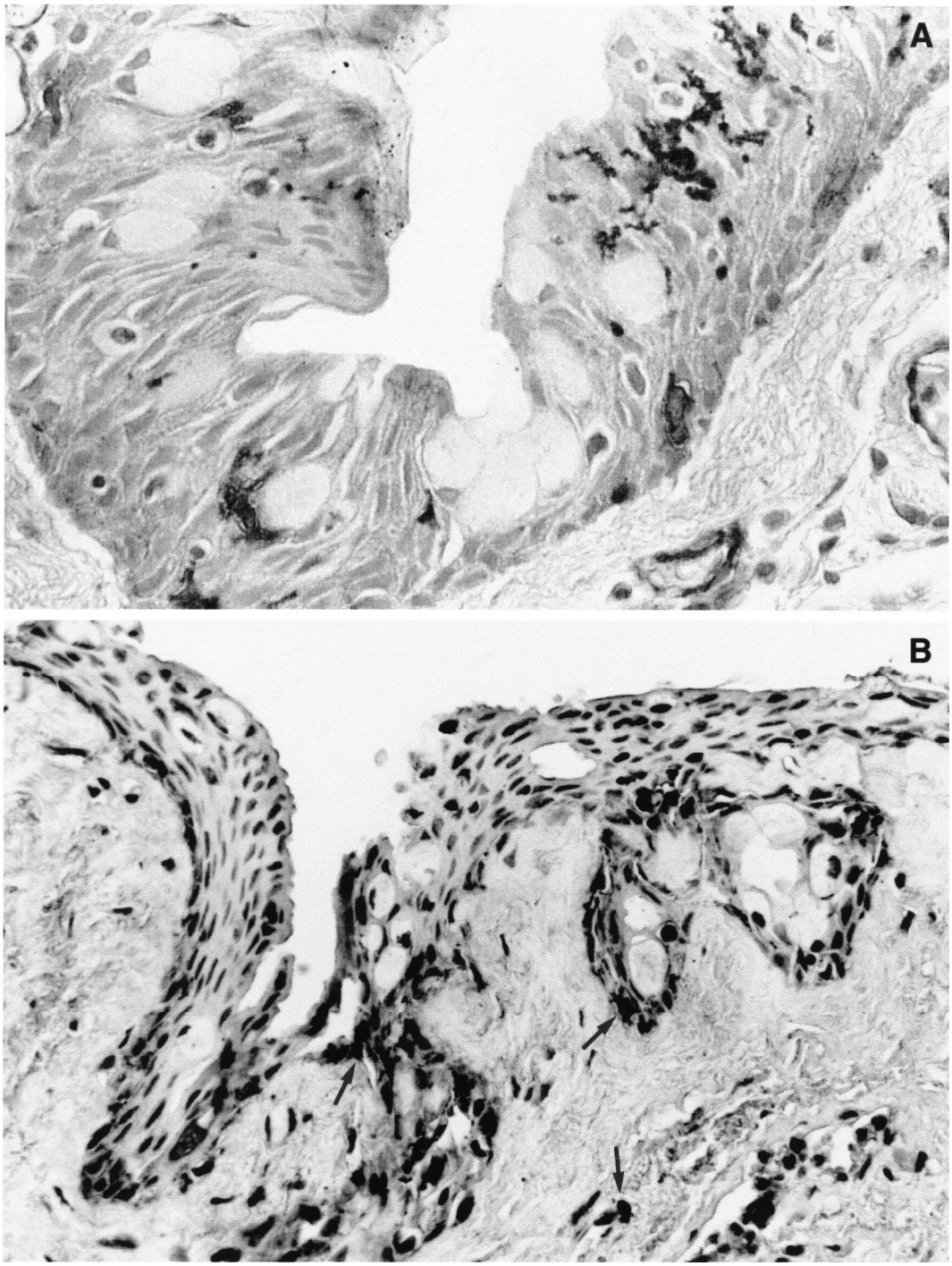

Fig. 5. A. Anti-human HLA-DR. **B.** Anti-cow S100 Protein. **A.** HLA-DR expression is found in both the stroma and the epithelium. **B.** S100-positive cells are present in the epithelium and in the substantia propria (arrows). A, x 630; B, x 400

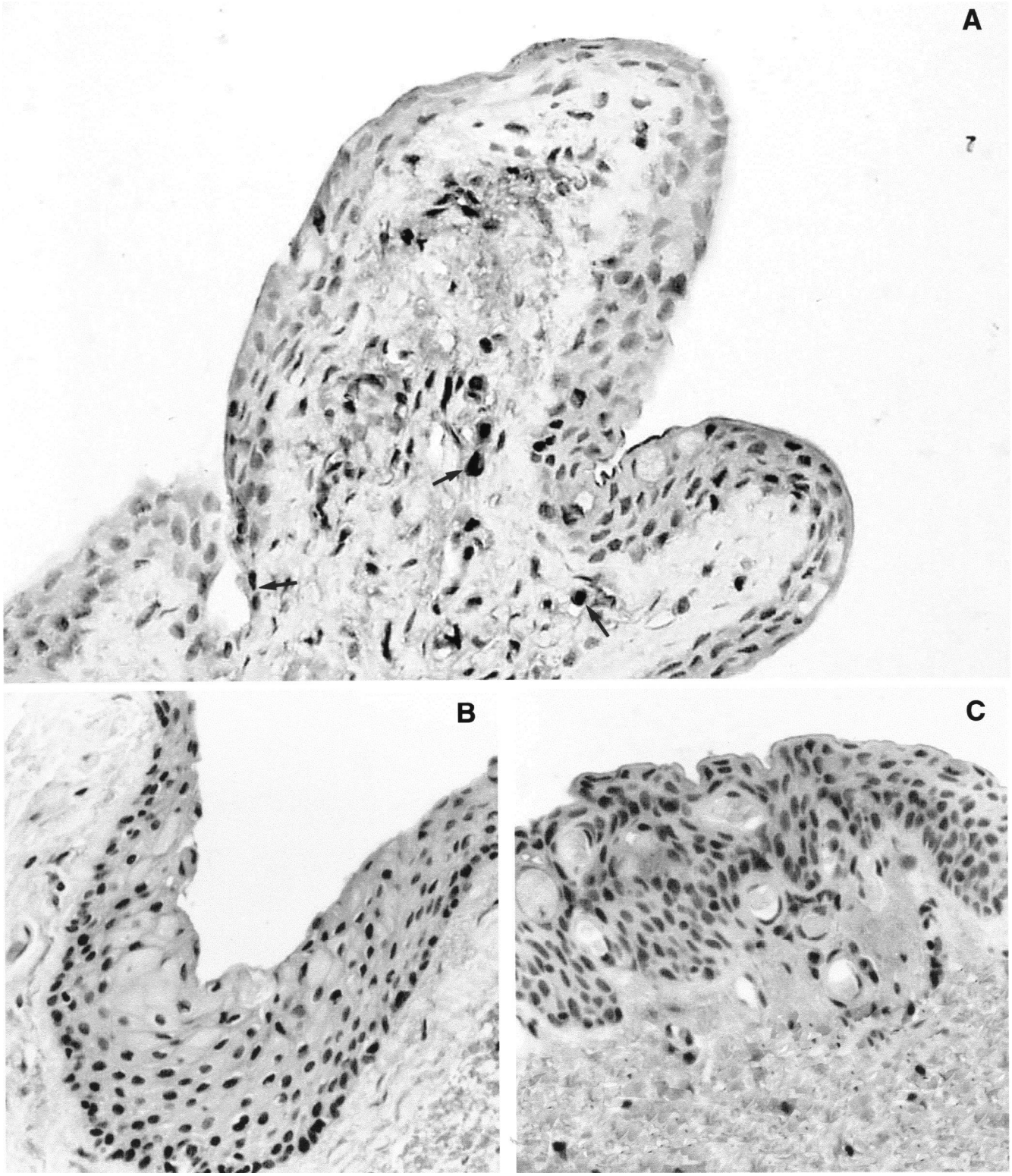

Fig. 6. A. Anti-cow S100 Protein. **B.** Anti-human HMB45**. C.** Anti-human Melan A. **A.** Only S100 shows immunoreactivity (arrows). x 400

immunoglobulin A (IgA), are thought to provide the ocular surface, as well as other mucus membrane areas of the body, with a first line of defense against microbial invasion (Tomasi and Plaut, 1985).

There are a variety of theories concerning the origin and the pathogenesis of pterygium. Histopathologically, the increased infiltration of lymphocytes, predominantly that of T-cells, plasma cells, and mast cells are observed; in addition, depositions of IgE and IgG have been reported (Pinkerton et al., 1984). Therefore, it has been suggested that an immunological mechanism involving hypersensitivity contributes to the pathogenesis of pterygium (Pinkerton et al., 1984; Liu and Yang, 1993).

Our data on pterygium lesions confirm previous reports that there are some immunoglobulins in the epithelial layer and in the substantia propria (Pinkerton et al., 1984; Liu and Yang, 1993). The authors examined pterygial tissues for IgG, IgE, IgA and IgM by direct immunofluorescence. While Pinkerton et al. failed to detect IgA and IgM, probably because of the low sensibility of the method they employed, indicating the specific presence of IgG only in the connective tissue stroma of the pterygium, corresponding to the area of plasma cells and lymphoid infiltration, Liu and Yang found deposition of immunoglobulins, especially in a granular pattern, around the basement membrane of the epithelial layer. In any case, these findings provided evidence that hypersensitivity takes part in the development of pterygium.

In the present study we indicate that IgA-, IgM- and IgG-positive immunocompetent cells were observable in the subepithelial stroma of the pterygium and an evident immunoreactivity for secretory IgA, IgG and IgM was present in the apical cells of the epithelium.

The immunoreactivity detected in the lamina propria suggests that IgA and IgM could be of local origin, as a

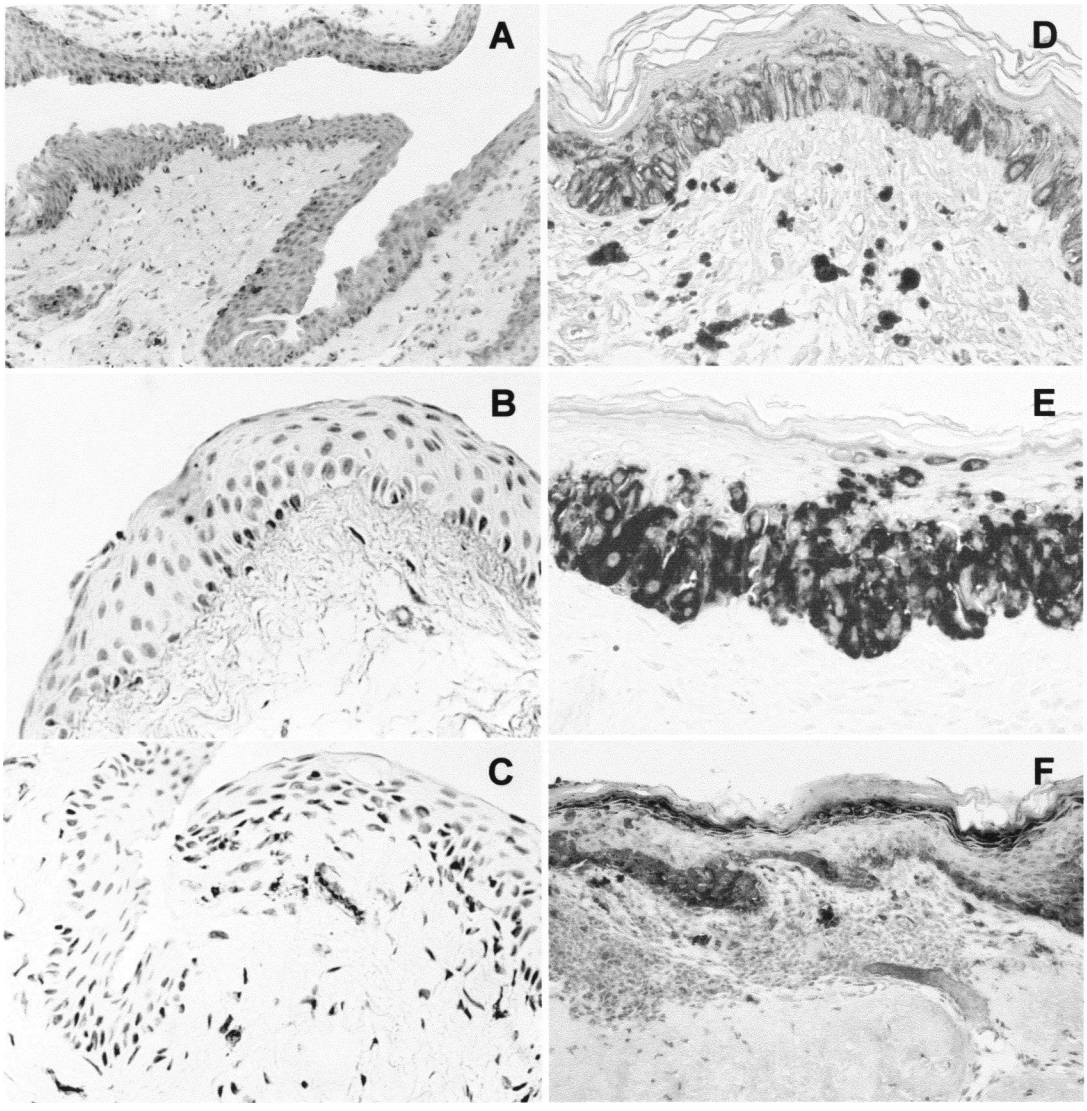

Fig. 7. Normal human conjunctiva and human melanoma. **A and D.** Anti-cow S100 Protein. **B and E.** Anti-human HMB45. **C and F.** Anti-human Melan A In the conjunctiva, only S100 shows immunoreactivity **(A)**. In the sections of melanoma a marked immunostaining is observed **(D, E, F)**. A, F, x 200; B, C, D, E, x 400

result of active secretion or of transudation. Moreover, although most of the IgG present in mucosal secretions is due to transudation from serum, virus-specific IgG antibodies produced by the mucosa can also contribute to total antiviral activity in mucosal secretions (Underdown and Mestecky, 1994).

The presence of lymphocytes and plasma cells in the stroma of the pterygial tissues indicated that an immunological process might be involved in the pathogenesis of this lesion. Moreover, the appearance of lymphocytes and plasma cells suggests that pterygium formation involves a chronic type of inflammation associated with the infiltration of mononuclear cells in the stroma. The presence of plasma cells and immunoglobulins in the lesion suggests that complement-mediated immunity also plays a role in the development of pterygium.

The characterization of the effector cells of the

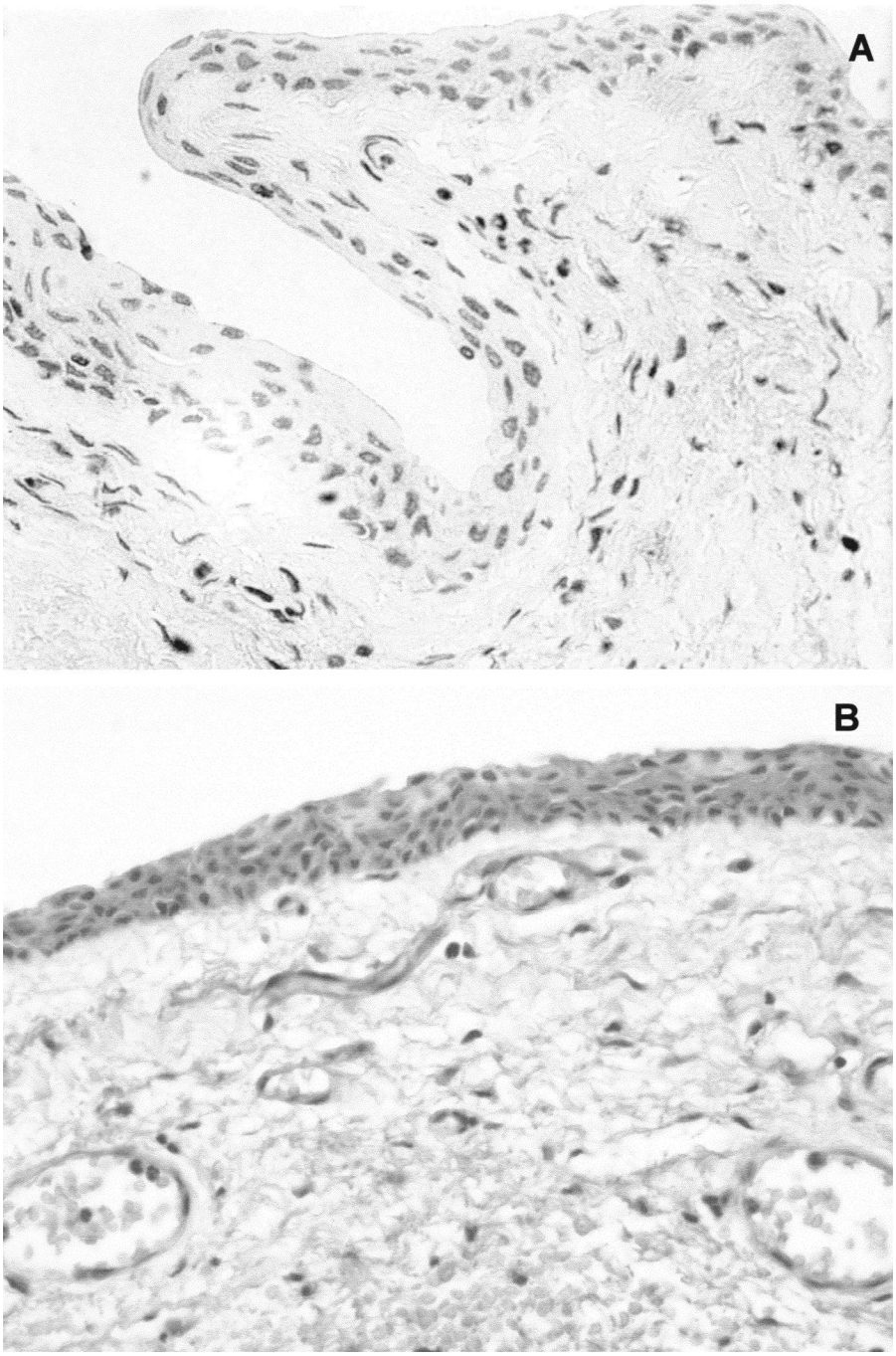

Fig. 8. A. Anti-human secretory IgA control section. **B.** Anti-human CD20 control section. **A and B.** The immunostaining has been completely abolished. x 400

immune system in diseased tissues, like pterygial tissues, is important in the study of the role of these cells in the development of the lesions. In a recent work of Ioachim-Velogianni et al. (1995) HLA-DR antigen expression in epithelial cells, B-cells, suppressor and helper lymphocytes, Langerhans' cell, and monocytes/macrophages were studied immunohistochemically in frozen sections of conjunctival specimens from patients with pterygium. In that study HLA-DR antigen expression was found to be closely related to the density of T-cells and, especially, of CD4 lymphocytes.

In agreement with previous reports, in the present study we have defined the distribution of the immunocompetent cells in a conjunctival lesion, like pterygium, in which a lymphocytic infiltration consisted predominantly of T-cells. Moreover, a great density of S100-positive cells, identified as Langerhans' cells (Tsironi et al., 2001), was noted, these cells playing a key role in the primary immune response because of their role as initiators.

Conjunctiva being considered both a mucosal inductive and effector site (unpublished data), an immunopathogenetic mechanism seems to be responsible for the pathogenesis of pterygium, perhaps being caused by pre-existing conjunctivitis or microtrauma in combination with the patient's predisposition; exogenous factors may contribute to the full development of the disorder.

Recent studies demonstrated that pterygium, a precursor of a precancerous condition such as actinic keratosis, often display the loss of heterozygosity (LOH) linked to the chromosomal region 9p21 (Detorakis et al., 1998), as well as melanoma (Fountain et al., 1992; Holland et al., 1994; Isshiki et al., 1994; Walker et al., 1994; Healy et al., 1996; Greene, 1999; Birindelli et al., 2000). This phenomenon indicates that also in pterygium, tumour suppressor genes could be involved in the development of such a lesion, and that a correlation between the two diseases could exist. Moreover, our study demonstrates that among the most sensitive markers for melanoma, only S100 shows immunoreactivity, suggesting that pterygium could be a progressive lesion with malignant biological behaviour not correlated to melanoma.

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