IMMUNOLOGY OF TRACHOMATOUS CONJUNCTIVITIS

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ABSTRACT

Trachoma, a chronic follicular conjunctivitis caused by infection with *Chlamydia trachomatis*, is a leading cause of preventable blindness. The blinding complications are associated with progressive conjunctival scarring. Our immunohistochemical studies of conjunctival biopsies from children with active trachoma demonstrated the presence of both humoral and cell-mediated immune responses. Antichlamydial antibodies can neutralize *Chlamydiae*, block attachment and internalization of the organism, and can produce partial immunity. Our observations suggest a role for T-lymphocytes and cell mediated immunity in the genesis of conjunctival scarring. Conjunctival epithelial cells expressed major histocompatibility complex (MHC) class II antigens which might allow conjunctival epithelial cells to present Chlamydial antigens to T-cells enhancing the immune response. The epithelial cells expressing MHC class II antigens might present autoantigens to T-cells leading to induction of an autoimmune reaction. We have demonstrated that the conjunctival epithelial cells from patients with trachoma expressed interleukin (IL)-1α and IL-1β. In addition, we have detected cytoplasmic expression of IL-1α, IL-1β, tumor necrosis factor α and platelet-derived growth factor by macrophages. These cytokines have the potential to influence the remodeling and fibrosis observed in trachoma. Alterations of extracellular matrix components and collagen metabolism occur in the conjunctival tissue from patients with trachoma. New collagen type V formation was noted in active trachoma and scarred trachoma. The conjunctival tissue from patients with active trachoma contained increased amounts of collagen types I, III and IV. Scarred trachoma is characterized by marked increase in basement membrane - collagen IV and marked decrease in collagen types I and III. In addition, we demonstrated increased activity of gelatinase B and numbers of inflammatory cells containing gelatinase B in trachoma patients suggesting that this enzyme might be involved in matrix degradation and promotion of conjunctival scarring in trachoma.

SAMENVATTING:

Trachoma is een van de frequentste oorzaken van voorkoombare blindheid. Het is een chronische folliculaire conjunctivitis die tot blindheid kan leiden door progressieve schrompeling van de conjunctiva. Onze immunohistochemische studies van conjunctiva biopsies bij kinderen met actief trachoma hebben zowel humorale als cel gebonden immuun reacties aangetoond. Antichlamydia antilichamen kunnen Chlamydia neutraliseren, ze kunnen de adhesie en absorptie beletten en zo een gedeeltelijke immuniteit geven. Onze observaties suggereren dat T-lymphocyten en cel gebonden immuniteit een rol spelen in de schrompeling van de conjunctiva. De epitheelcellen van de conjunctiva tonen HLA type II antigenen zodat deze epitheelcellen Chlamydia antigenen kunnen aanbieden aan de T-cellen en zo de immuun reactie aanwakkeren. De epitheel cellen kunnen ook met hun HLA type II complexen autoantigenen aanbieden aan de T-cellen en zo een autoimmuun proces induceren. Wij toonden aan dat conjunctivale epitheel cellen van trachoma lijders interleukin (IL)-1α en IL-1β aanmaken. Wij toonden ook dat macrophagen in hun cytoplasma IL-1α en IL-1β, tumor necrosis factor α en bloedplaatjes groei factor bezitten. Deze cytokines kunnen een rol spelen in de veranderingen en de fibrose van de conjunctiva in trachoma. Bij deze patiënten zien we aantasting van de extracellulaire matrix en van het collageen metabolisme. Collageen type I, III, IV ziet men bij patiënten met actief trachoma. Collageen V vindt men in actief zowel als in cicatricieel trachoma. In dit laatste stadium ziet men een sterk verdikte basaal

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membraan, collageen IV en een sterke vermindering van collageen I en III. Ook toonden wij een verhoogde activiteit van collagenase B en talrijke ontstekingscellen die collagenase B bevatten, hetgeen suggereert dat dit enzyme kan bijdragen tot de ontaarding van de matrix en de bevordering van de fibrose in trachoma.

RÉSUMÉ

Le trachome, une conjonctivite folliculaire due à une infection par Chlamydia trachomatis, est une cause majeure de cécité évitable. Les complications qui mènent à la cécité sont associées à la cicatrisation progressive de la conjonctive. Nos recherches immunohistochimiques faites sur des biopsies conjonctivales chez des enfants souffrant de trachome au stade actif, montrent la présence de réponses immunologiques humorales et cellulaires. Les anticorps antichlamydiens sont capables de neutraliser les Chlamydias, de bloquer leur adhérence et leur absorption et de donner ainsi une immunité partielle. Nos observations suggèrent un rôle pour les lymphocytes-T et l'immunité cellulaire dans la genèse de la fibrose conjonctivale. Les cellules épithéliales de la conjonctive expriment les antigènes du complexe majeur d'histocompatibilité de classe II ce qui permet à ces cellules de présenter aux cellules T les antigènes chlamydiens ainsi que des auto-antigènes ce qui peut induire une réaction auto-immunitaire. Nous avons pu détecter, chez les trachomateux, l'expression de l'interleukine (IL)-1α et IL-β par les cellules épithéliales de la conjonctive ainsi que l'expression cytoplasmique par les macrophages de cette même interleukine, du facteur de nécrose tumorale α et du facteur de croissance associé aux plaquettes. Ces cytokines peuvent influencer la fibrose et le remodelage observé en cas de trachome. Le tissu conjonctival des trachomateux montre des altérations des composants extra-cellulaires et du métabolisme du collagène. La formation du collagène type V s'observe dans le trachome actif et cicatriciel. La conjonctive de patients avec un trachome actif montre des quantités accrues de collagène I, II et IV, tandis que le tissus sous-conjontival dans le trachome cicatriciel montre une diminution marquée du collagène I et III et un épaississement net de la membrane basale -collagène IV. En outre nous avons montré une activité accrue de la collagénase B et du nombre de cellules inflammatoires contenant cet enzyme. Ceci suggère que la collagénase B pourrait être une cause de la dégradation du tissu sous-conjonctival et de la fibrose de la conjonctive atteinte de trachome.

KEY-WORDS:

Conjunctiva, trachoma, Chlamydia, cytokines, collagen, matrix metalloproteinases, major histocompatibility complex antigens.

MOTS-CLÉS:

Conjonctive, trachome, Chlamydia, cytokine, collagène, métalloprotéinase, CMH.

INTRODUCTION

Trachoma is a chronic follicular keratoconjunctivitis caused by *Chlamydia trachomatis* serotypes A, B, Ba and C (96). It remains a major worldwide cause of preventable blindness and a major public health problem, particularly in the Third World. It accounts for some 25% of world blindness, and is the commonest infectious cause of blindness (103). An estimated 500 million people have had the disease and 7 to 9 million of these have lost vision, mainly as a result of corneal opacification (22). Its severe effects are especially prominent in the developing countries of Africa, the Middle East, Southeast Asia and the subcontinent of India, and there are pockets of disease in Latin America, the Pacific Islands and the Australian outback (100). It presents serious obstacles to socioeconomic development and human productivity and consumes significant human and material resources allocated to the care of those who are blinded by it.

Chlamydiae are obligate intracellular pathogens that resemble gram-negative bacteria in some respects but differ primarily by the lack of energy-yielding metabolic pathways. They lack the ability to synthesize high-energy compounds such as adenosine triphosphate (ATP) and guanosine triphosphate (GTP). These compounds, essential for metabolism and respiration must be provided by the infected host cell. Thus Chlamydiae have been called ''energy parasites'' (83). *Chlamydiae* demonstrate two distinct stages within their developmental cycle (110). The infectious form, the 200-300 nm elementary body, is metabolically inactive, and has a dense DNA core surrounded by a trilaminar cytoplasmic membrane, external to which is a trilaminar outer envelope. The elementary body is phagocytosed by the host cell after specifically binding to the surface of epithelial cells. Within 6-9 hours after infection, the elementary body becomes metabolically active and enlarges (1 µm) to form an initial or reticulate body. The reticulate body multiples by binary fission to form a microcolony of pleomorphic Chlamydial forms which lies within a cytoplasmic vacuole. By 20 hours after infection some of the reticulate bodies undergo reorganization within the expanding intracytoplasmic inclusion body of Halberstaedter-Prowazek (Figures 1 and 2) into smaller intermediate forms and then into elementary bodies. The new elementary bodies are released by cell lysis to infect new cells.

Fig. 1 Conjunctival scraping from a child with active trachoma showing multiple epithelial cells containing intracytoplasmic Halberstaedter-Prowazek inclusion bodies (arrows) (Giemsa x 800).

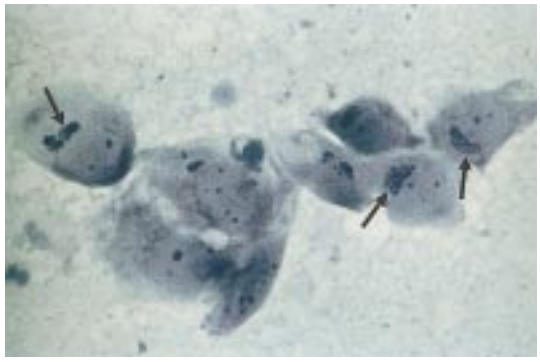
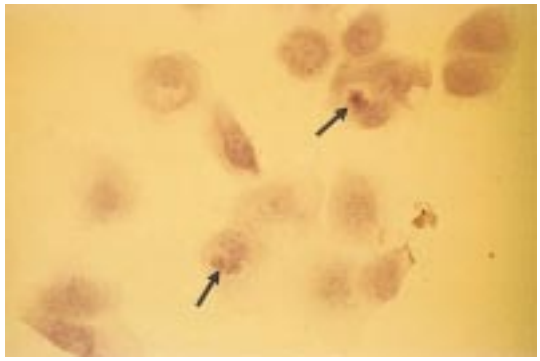


Fig. 2 Corneal epithelial cells from a child with active trachomatous keratitis obtained by corneal replica technique showing a Halberstaedter-Prowazek inclusion body (arrows) (Giemsa x 1120).



Over the last 10 years, much has been learned about the antigenic composition of *Chlamydiae*. Their rigid outer membrane is composed of a number of structural proteins, the most important being the major outer membrane protein (68). Antibodies against this protein are capable of neutralizing *Chlamydiae* (94) and can produce at least partial immunity to Chlamydial infection. In addition, T-lymphocyte proliferative responses and interferon-gamma (IFN-γ) production were observed after challenge with major outer membrane protein (90). Other prominent components of the outer membrane include a 60 KD cysteine-rich protein and a spe-

cific Chlamydial lipopolysaccharide (LPS). *Chlamydiae* also release two heat-shock proteins, of 57 KD and 75 KD. The 57 KD heat-shock protein appears to be the major pathogenic antigen in trachoma. It stimulates the persistent deleterious cell-mediated immune response which, if chronically maintained, leads to cytokine release, causing ongoing inflammation, fibrosis and conjunctival scarring (21,62,73).

In the early (active) stage, which is seen principally among children in endemic areas, trachoma is characterized by a chronic follicular conjunctivitis, which affects principally the upper tarsal conjunctiva, but also the limbus. Conjunctival thickening and a papillary response may also be seen with active inflammation, especially in the presence of bacterial secondary infection. Corneal changes include punctate keratitis, indolent ulcers and vascular and cellular infiltration (pannus), which is occasionally so extensive as to impair vision. Among older individuals in endemic areas, conjunctival scarring replaces the follicles and may lead to dry eye syndrome, entropion and trichiasis. Corneal scarring and blindness might result from regressed pannus and/or constant corneal abrasion due to entropion and trichiasis.

The pathologic mechanisms by which trachoma causes corneal and conjunctival scarring that lead to blindness are still unclear. Although the growth of *C. trachomatis* is restricted to the epithelium (43), the consequences of infection, namely conjunctival and subconjunctival scarring and blindness can be devastating. There is a growing evidence that the blinding complications of trachoma may result from chronic stimulation of the immune response. Monnickendam et al (61), using a guinea pig model of Chlamydial eye disease, demonstrated that repeated reinfection led to chronic conjunctivitis with pannus and conjunctival scarring. Ocular infection with the agent of *C. trachomatis* induces both humoral immunity (74,104) and cell-mediated immunity (51,62,118). The immune response seems to confer partial protection against subsequent infection, yet appears also to be responsible for much of the observed pathology and tissue destruction seen in trachoma (99).

PHENOTYPIC CHARACTERIZATION OF INFLAMMATORY CELLS IN TRACHOMATOUS CONJUNCTIVITIS

Conjunctival biopsy specimens from children with severe active trachoma showed comparable changes. The epithelium showed mild to moderate hyperplasia and was infiltrated by a mixed inflammatory infiltrate consisting of mononuclear and polymorphonuclear leucocytes. In the superficial epithelial layers variable numbers of Halberstaedter-Prowazek inclusion bodies were seen. In the underlying substantia propria, the inflammatory infiltrate was organized in lymphoid follicles and as a diffuse infiltrate. Variably sized secondary lymphoid follicles containing a large, clear follicular centre surrounded by a lymphocytic mantle were seen throughout the biopsy specimens. The follicular centre was composed of small and large activated cleaved and non-cleaved lymphoid cells, and contained many stainable body macrophages. The surrounding lymphocytic mantle was thinned and contained small darkly stained lymphocytes and lymphocytes of variable size and shape, indicating a progressive transformation. In the area between the follicles and the epithelium, as well as in areas of diffuse inflammation, a mixed population of lymphocytes, polymorphonuclear leucocytes, macrophages and some mast cells and eosinophils was observed. The blood vessels in these areas were distended and lined by prominent endothelium. A band of plasma cells was situated directly underneath the epithelium and a dense infiltration by plasma cells was observed also around the acini of accessory lacrimal glands. Immunohistochemical staining demonstrated that all epithelial cells expressed β_2 -microglobulin (class I major histocompatibility complex (MHC] associated); membranous MHC class II antigen expression was noted mainly in the superficial epithelial layers (Fig. 3). The inflammatory infiltrate in the epithelium consisted of large numbers of $3MA134^+$ macrophages, as well as variable numbers of MT_1^+ T-lymphocytes and polymorphonuclear leucocytes.

MHC class II^+ dendritic cells were observed in the deeper epithelial layers as well as in the

Fig. 3
Immunohistochemical staining showing membranous major histocompatibility complex class II expression in the superficial epithelial layer (x 385).

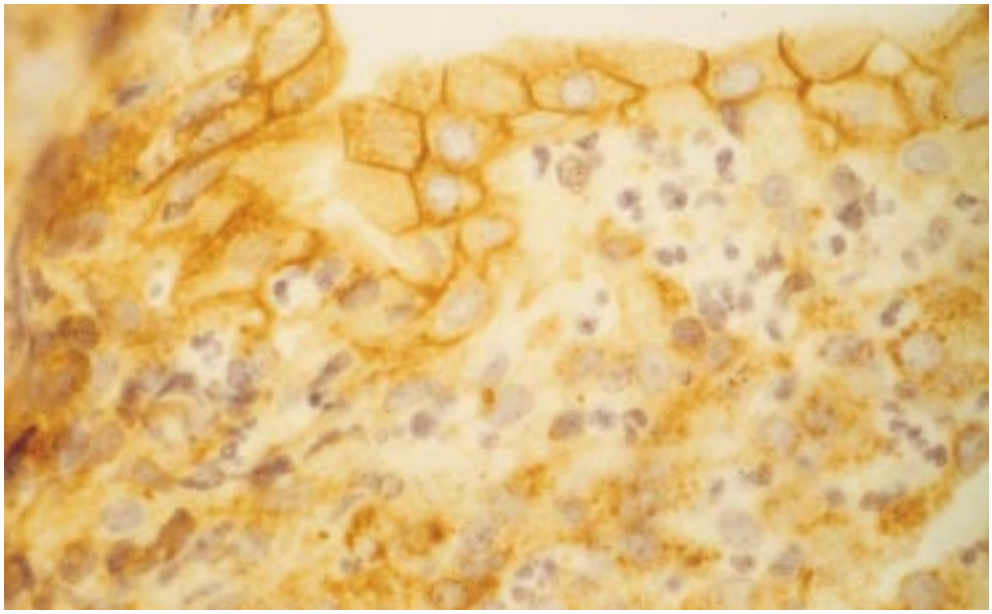


Fig. 4
Immunohistochemical staining showing that the lymphoid follicle and surrounding mantle are composed mostly of B-lymphocytes (x 135). (Reproduced with permission from reference 7)

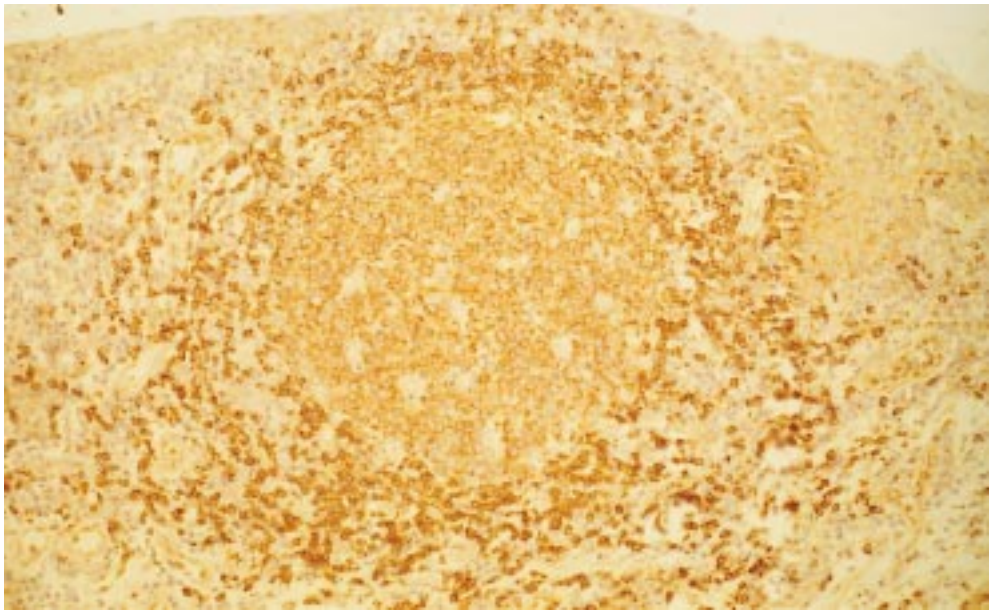


Fig. 5
Immunohistochemical staining showing large immunoreactive macrophages in the follicular centre. Smaller macrophages are present also in the surrounding infiltrate and in the epithelium (x 135). (Reproduced with permission from reference 7)

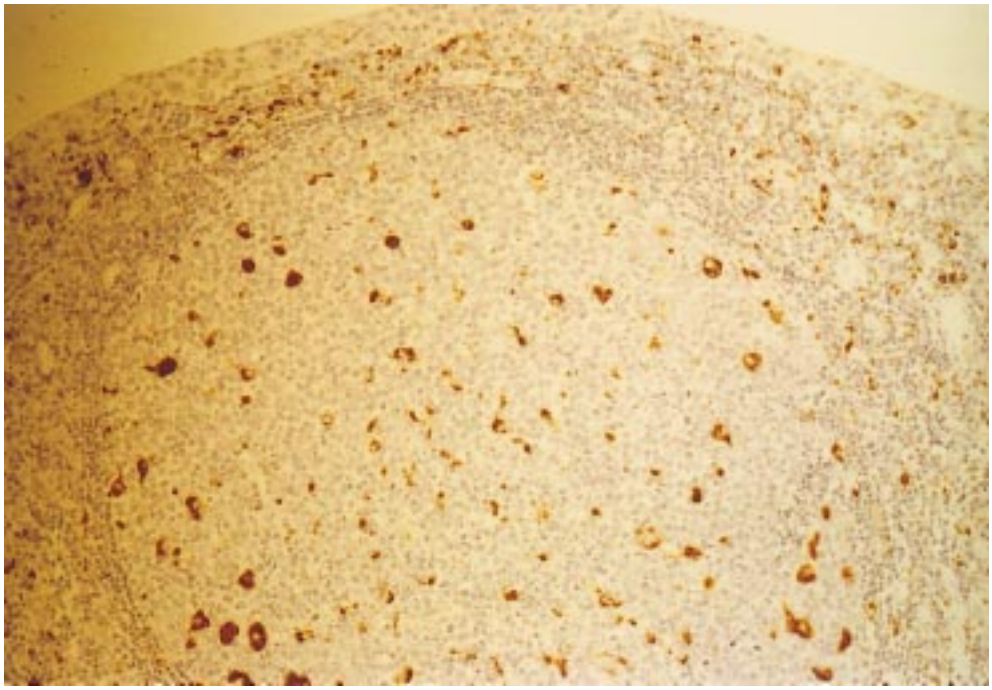
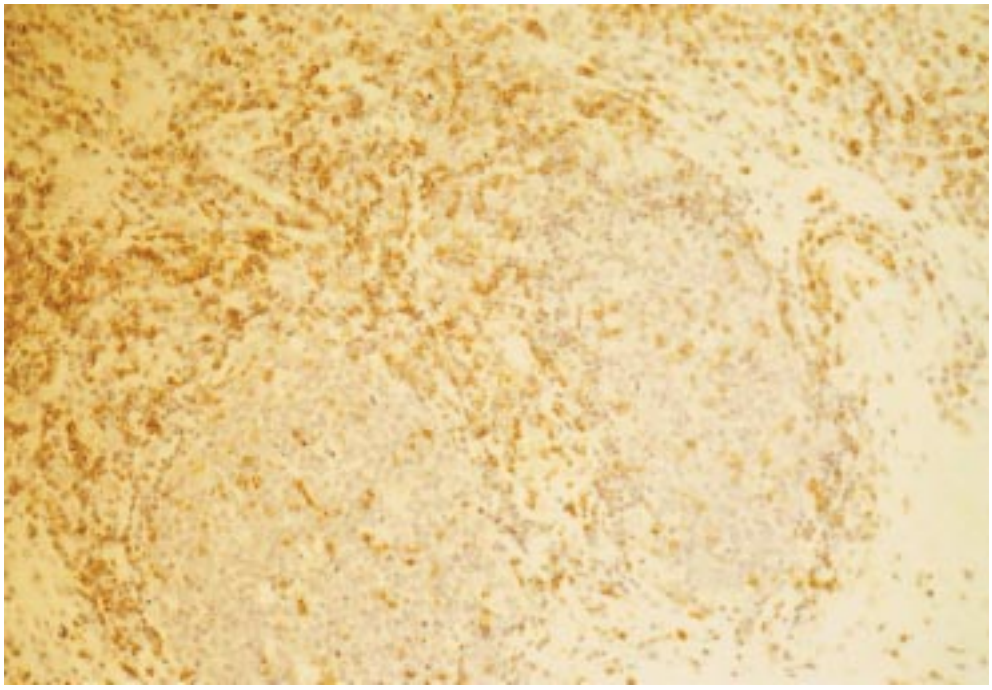


Fig. 6
Immunohistochemical staining showing few T-lymphocytes in the follicular centre, whereas many T-lymphocytes are present in the surrounding infiltrate (x 135.)



underlying substantia propria, where they were admixed with MT_1^+T- lymphocytes, $3MA134^+$ macrophages and scattered MB_2^+ lymphoid cells. Occasional dendritic cells were situated at the interface between epithelium and substantia propria. The lymphoid follicles consisted of MB_2^+ LN_2^+ MHC class II^+ B-lymphocytes (Fig. 4). In the follicular centre large $3MA134^+$ stainable body macrophages (Fig. 5) and some MT_1^+ T-cells were observed (Fig. 6). In the subepithelial band of plasma cells IgA^+ cells outnumbered IgG^+ cells, whereas IgM^+ and IgE^+ plasma cells were rare (Fig. 7). A similar Ig distribution was found in the plasma cells populating the accessory lacrimal glands (Fig. 8) (1,7).

These results indicate the presence of distinct compartments involved in humoral and cell mediated immune responses. The humoral immune response was represented by large, active lymphoid follicles formed of B-lymphocytes, scattered transformed B lymphoid cells in the lymphocytic mantle and area between the follicle and epithelium, and large numbers of plasma cells below the surface epithelium and around the acini of accessory lacrimal glands. Previous in vitro studies (49) have shown that *C. trachomatis* stimulates B-cells to proliferate and differentiate into immunoglobulin-secreting plasma cells. Differentiation to plasma cells is enhanced by T-cells, leading to the secretion of large quantities of polyclonal immunoglobulins. Our results indicate that the in vivo counterpart of this in vitro response consists of B-cells that are stimulated by Chlamydial antigens exposed on the dendritic reticulum cells acting as antigen presenting cells in the follicular centre. Subsequently the B- cells undergo transformation, leave the follicular centre, and differentiate into plasma cells, which migrate through the region between the lymphoid follicles and the epithelium. That region was found to consist mainly of T-cells and dendritic cells, and it thus appears to represent a suitable microenvironment for the terminal differentiation of B-lymphocytes into plasma cells. IgA^+ plasma cells outnumbered IgG^+ plasma cells, whereas IgM^+ and IgE^+ plasma cells were conspicuously rare. These findings are in accordance with the detection of type-specific Chlamydial IgA and IgG antibodies in the tears of patients with trachoma (104). Dense accumulations of plasma cells, showing a similar Ig distribution, were seen around the acini of accessory lacrimal glands, suggesting that these glands constitute part of the conjunctival protective immune system. According to Franklin and Remus (29) these IgA^+ plasma cells originate from B-cells present in the conjunctiva-associated lymphoid tissue (CALT) and are involved in the formation of secretory IgA, which has been found to play a part in the defence against Chlamydial infection by preventing its adherence to epithelial cells (23). It has been demonstrated that antichlamydial antibodies are capable of neutralizing chlamydiae, blocking attachment and internalization of the organism, and can produce partial immunity (94,112).

Scattered MHC class II^+ dendritic cells were observed in the epithelium, in the underlying substantia propria and at the border of both regions. By analogy with the skin (87) these dendritic cells might be antigen-loaded Langerhans cells, previously shown to occur in the conjunctival epithelium (30,31) which have migrated towards the underlying substantia propria, where they present their antigens in an MHC class II-restricted manner to T- lymphocytes. In vitro studies have shown that antigen presenting cells are able to process and present Chlamydial antigens to T-lymphocytes inducing T-cells proliferation. These T- cell proliferative responses were major histocompatibility complex restricted (95). In addition, Stagg et al (90) have demonstrated T-cell proliferative response and interferon-gamma production to major outer membrane protein of *C. trachomatis*. This response displayed an absolute requirement for dendritic cells in the antigen-presenting cell population. In addition to helper T-cells involved in B-cell differentiation, the antigen-specific proliferation of T-cells induces the development of cytotoxic effector T-cells (92). The majority of T-lymphocytes present in the epithelium are likely to belong to the cytotoxic T-cell subset engaged in cytotoxicity of infected cells. For the cytotoxic T-cells to mediate cytotoxicity they must recognize class I MHC antigen displayed by diseased cells.

We have demonstrated that CD_4^+ T-lymphocytes were greater in number than CD_8^+ T-lymphocytes (4) (Fig. 9) which is similar to that described in previous reports of inflamed and

Fig. 7
IgA staining showing many IgA^+ plasma cells in a subepithelial band (x 135). (Reproduced with permission from reference 7)

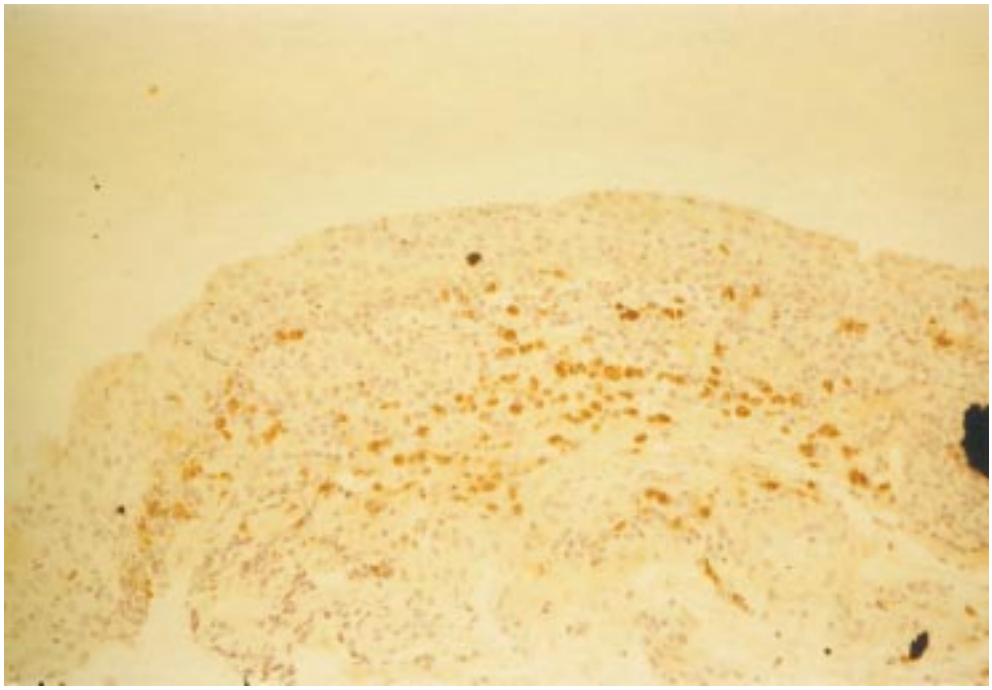


Fig. 8
IgA staining showing dense accumulation of IgA^+ plasma cells around the acini of accessory lacrimal glands (x 135).

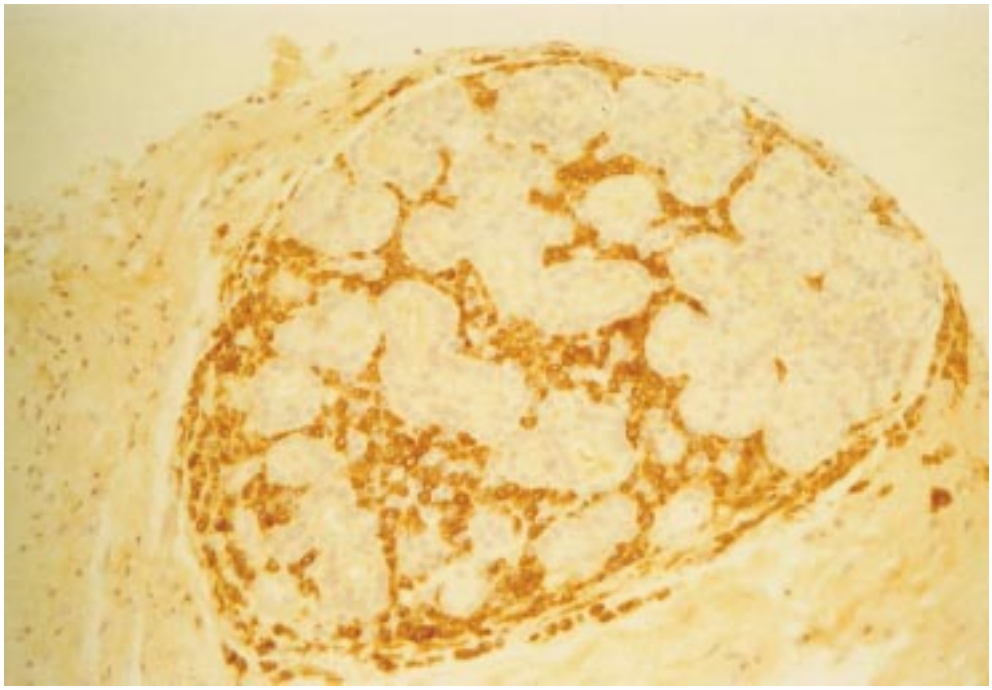
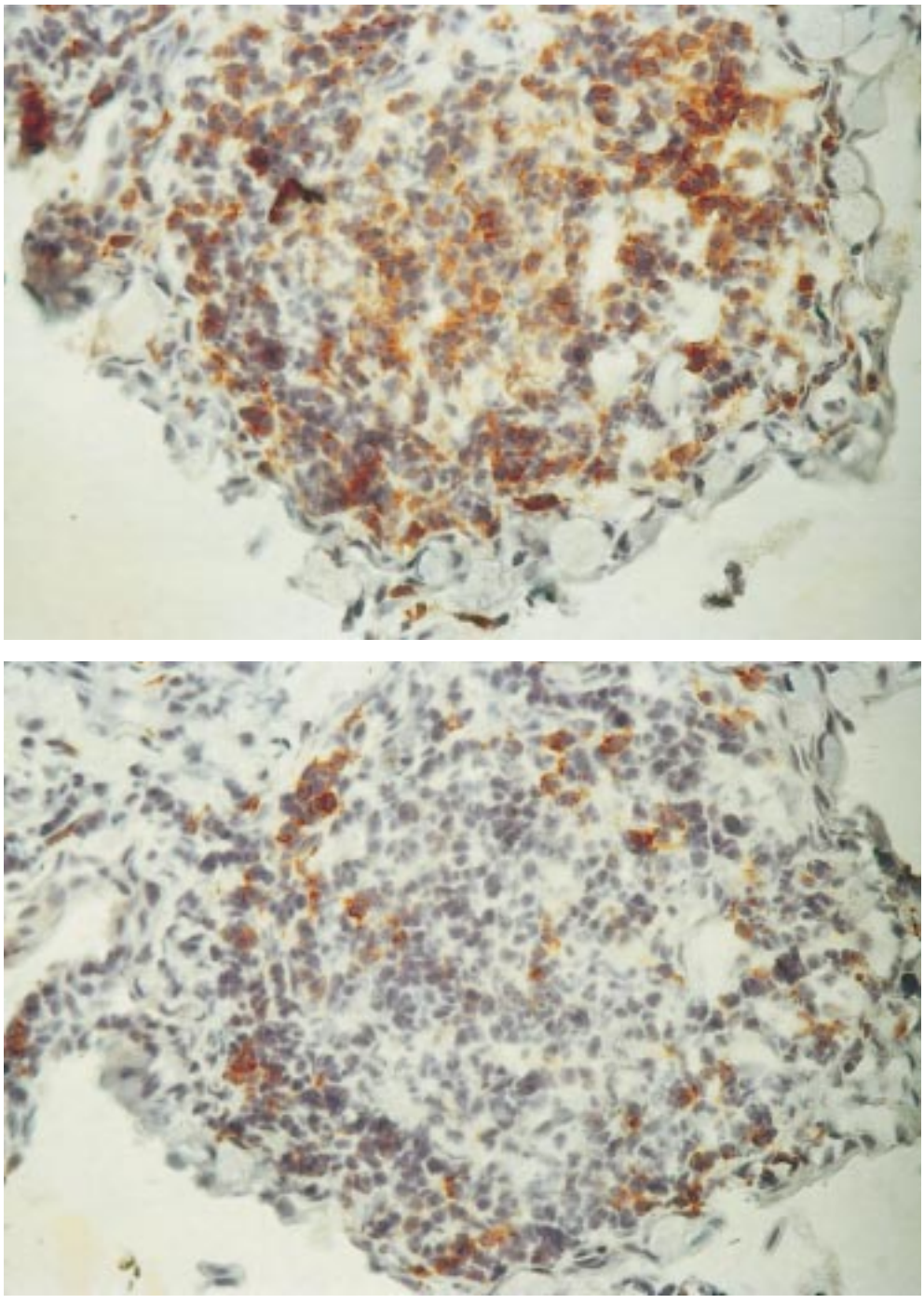


Fig. 9
Serial sections immunohistochemical stainings for CD4 (top) and CD8 (bottom). Note that $CD4^+$ helper / inducer T-lymphocytes predominated over $CD8^+$ suppressor / cytotoxic T-lymphocytes (300).



active trachoma (16,77). Two different types of CD_4^+ T-cell clones have been identified on the basis of their differing cytokine profile (63). T helper (TH) 1 cells produce IL-2 and IFN-γ and mediate several functions associated with cell-mediated immunity, while TH2 cells produce IL-4, IL-5, IL-6 and IL-10 and are more effective in stimulating B- cells to produce antibody. Previous studies have demonstrated that cellular immune responses in the local tissues associated with Chlamydial infections are dominated by TH1-like responses (18,89) and intravenous transfer of *C. trachomatis*-specific TH1 clone induced resolution of murine Chlamydial genital infection (41). In our immunohistochemical studies of conjunctival specimens from patients with active trachoma, we have demonstrated that the epithelial cells expressed MHC class II antigens (1,7). It is well known that the expression of MHC class II antigens requires stimulation with IFN-γ (6) produced by activated TH1 cells. Based on these findings we may assume that the large preponderance of CD_4^+ T-cells observed in the conjunctival biopsies comprised TH1 cells. Igietseme et al (40) have demonstrated that CD_8^+ T-cells may contribute to antichlamydial T-cell immunity in vivo. In addition, Starnbach et al (91) characterized a cytotoxic T-lymphocyte line derived from mice infected with *C. trachomatis*. This cytotoxic T-lymphocyte line is specific for, and able to lyse, Chlamydia-infected cells, and recognizes a peptide epitope present on infected cells in the context of MHC class I molecule. Based on these observations cytotoxic T-cells could comprise the major component of CD_8^+ T-cells detected in conjunctival specimens.

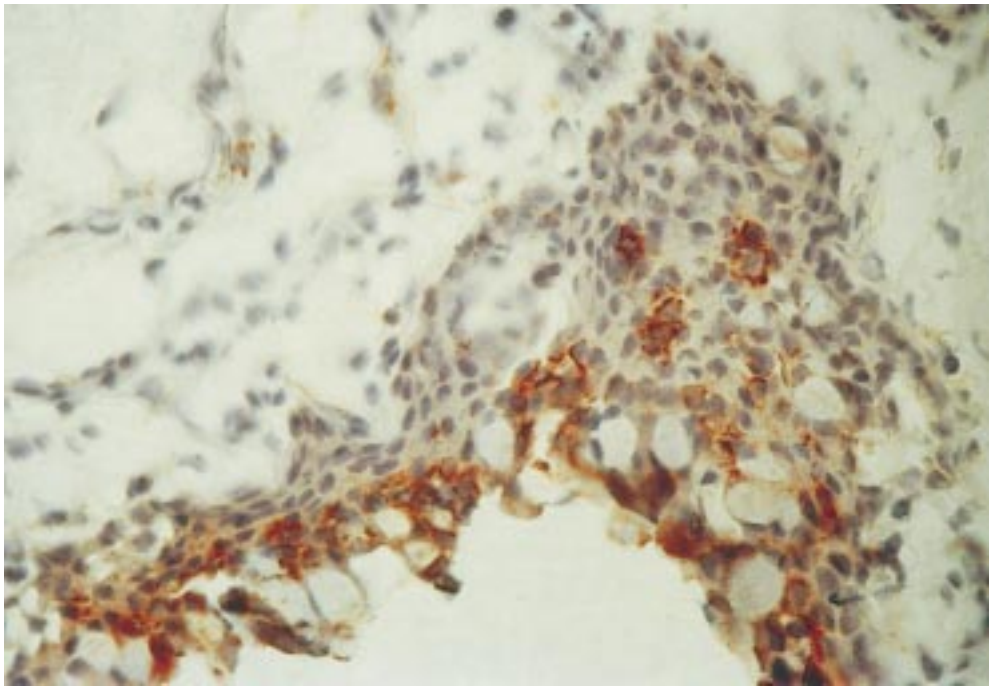
In an immunohistochemical study of conjunctival biopsies from children with mild to moderate active trachoma (4), lymphoid follicles were present in six out of nine conjunctival specimens. In these specimens the number of B-lymphocytes was greater than T-cells and the fibrosis was confined to the deep substantia propria. In three specimens lymphoid follicles were not detected and T-lymphocytes outnumbered B-cells. In these specimens fibrosis was more pronounced and involved the whole substantia propria, starting immediately under the epithelium. Our findings are supported by previous studies (16,77) of lymphocyte subsets in adults with cicatricial trachoma that demonstrated predominance of T-lymphocytes over B-cells. These observations suggest a role for T-lymphocytes in the genesis of conjunctival scarring in cicatricial trachoma. Several lines of evidence suggest that T-lymphocytes play a direct role in the pathogenesis of fibrosis (11,108,109). T-lymphocytes produce mediators including IL-2 and interferon-γ (IFN-γ) which trigger macrophage production of fibrogenic cytokines. Furthermore, T-lymphocytes produce transforming growth factor-β (TGF-β) which stimulates collagen synthesis (46). On the other hand, T-lymphocytes and cell-mediated immunity are essential for the eradication of Chlamydial infections (40,41,93,109). T- lymphocytes stimulation by *C. trachomatis* elementary bodies elicit IFN-γ production (28). IFN-γ produced by activated T-cells has been shown to inhibit the intracellular growth of *Chlamydia* (97) and participate in the defense against early infection (59). In addition, IFN-γ exhibits cytotoxic activity against cells infected with *C. trachomatis* (17). Therefore, activation of T cells could bring about both protective and deleterious effects.

Increased numbers of macrophages were noted in the conjunctival biopsy specimens from patients with active trachoma (4,7). Macrophages are important components of the immune response to foreign agents. It has been demonstrated, using an in vitro system, that macrophages inhibit intracellular *C. trachomatis* replication (56). In addition, macrophages play a central role in normal wound healing and pathologic fibrosis by virtue of their ability to release a variety of fibrogenic cytokines (46).

CONJUNCTIVAL EPITHELIAL CELLS INFECTED WITH CHLAMYDIA TRACHOMATIS EXPRESS MHC CLASS II ANTIGENS

The epithelial cells not only expressed the class I MHC antigens associated β_2 -microglobulin, but were also found to express MHC class II antigens at their surface (Fig. 3) (1,7). MHC class II antigens are present on cells involved in im-

Fig. 10
Anti-IL-1α staining shows cytoplasmic IL-1α expression in the superficial epithelial layers (x 300). (Reproduced with permission from reference 4)



mune responses, including B cells, activated T cells and antigen-presenting cells. The presence of MHC class II antigens on these cells is associated with antigen recognition and presentation to T cells and the initiation of specific B- and T-cell responses (102,105). We have shown that the normal conjunctival epithelium does not express MHC class II antigens (30). Its expression on the conjunctival epithelial cells in trachoma patients is probably due to the release of interferon-gamma by activated T-cells present in the epithelium. Interferon-gamma has been shown to induce MHC class II antigens synthesis and expression by a variety of epithelial cells in vitro (6,12,98) and is produced by lymphocytes stimulated with *C.* trachomatis (28). MHC class II antigens expression might allow conjunctival epithelial cells to present Chlamydial antigens to T-cells and thus to enhance the immune response in trachoma. Moreover, this MHC class II antigens expression may result in unopposed T-cell proliferation and lead to perpetuation of the immune response. In addition, induction of MHC class II antigens expression on epithelial cells has been associated with autoimmune reactions--for example, autoimmune thyroiditis (14,35) and diabetes mellitus (13). The epithelial cells expressing MHC class II antigens might present autoantigens to T-lymphocytes which activate effector B-and T-lymphocytes, leading to induction of an autoimmune reaction (50). In this context the appearance of autoantibodies in patients with Chlamydial salpingitis (9) may be relevant. Alternatively, expression of MHC class II antigens may render the infected epithelial cells susceptible to immunological attack by MHC class II-restricted cytotoxic T-cells (60). The expression of MHC class II antigens by the conjunctival epithelial cells may therefore contribute to the progression of conjunctival scarring in trachoma.

Fig. 11
Collagen V immunohistochemical staining of scarred trachoma showing thick, continuous immunoreactivity in upper substantia propria, around stromal vessels (top) and around acini of accessory lacrimal glands (bottom) (x 300). (Reproduced with permission from reference 5)

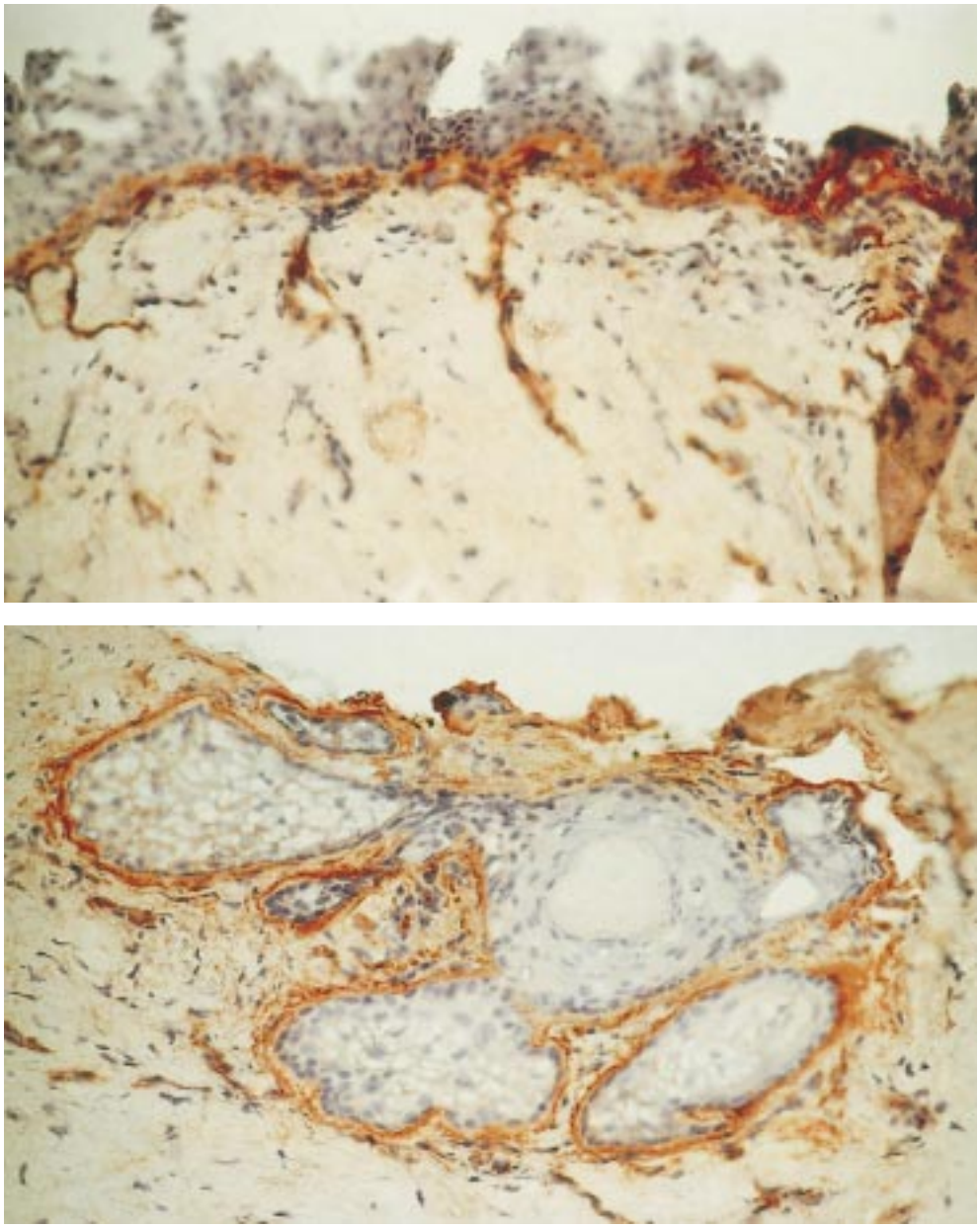
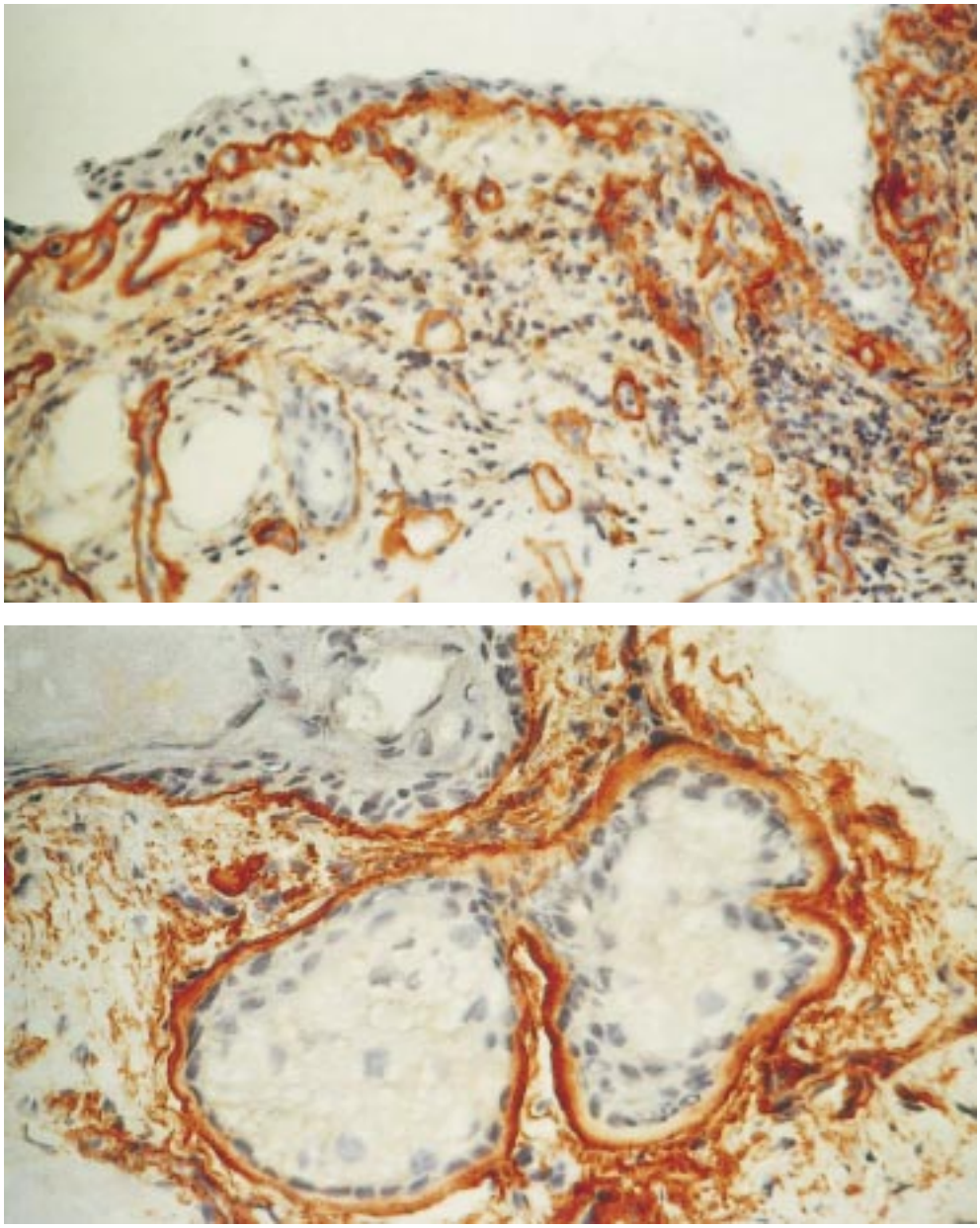


Fig. 12
Collagen IV immunohistochemical staining of scarred trachoma showing very thick, intensely stained epithelial basement membrane, thick vascular endothelial basement membrane (top) and very thick, intensely stained accessory lacrimal glands basement membrane (bottom) (x 300). (Reproduced with permission from reference 5)



EXPRESSION OF FIBROGENIC CYTOKINES IN TRACHOMATOUS CONJUNCTIVITIS

Cytokine production during infection may play an important role in modulating host defenses to *C. trachomatis*. This may also be a factor in the perpetuation of the inflammation and subsequent fibrosis. We have demonstrated that the conjunctival epithelial cells from patients with trachoma showed cytoplasmic expression of interleukin (IL)-1α and IL-1β (Fig. 10) (4). Normally, conjunctival epithelial cells do not express IL-1. These findings demonstrate the in vivo expression and the production of IL-1 by the conjunctival epithelial cells in trachoma. This production of IL-1 may constitute an important contributing factor in the process of scarring in trachoma. IL-1 is a pleiotropic cytokine produced chiefly by monocytes and macrophages but also by cells of epidermal, epithelial, lymphoid and vascular origin (25). Our results are consistent with the findings of Rothermel et al (79) that *C. trachomatis* induced IL-1 production by human blood monocytes. They showed that human blood monocytes produced detectable IL-1 when cultured with as little as 1 μg of Chlamydial protein per ml, which corresponded to 4 to 40 *Chlamydiae* per monocyte. This suggests that during low-grade or subclinical infections, which are characteristic of Chlamydial disease, very few organisms may be sufficient to stimulate IL-1 production. Furthermore, Magee and associates (52) have demonstrated increased m RNA and bioactivity for IL-1 in murine lungs after Chlamydial infection. The relevance of IL-1 to trachoma was also suggested by the detection of increased levels of IL-1 in the tears from children with active trachoma (79).

As an inflammatory mediator, IL-1 affects tissue remodeling by inducing the production of collagenase (24,36) and collagen (26). Furthermore, IL-1 can stimulate fibroblast proliferation (26,75,119). Excessive IL-1 production is thought to contribute to tissue damage and fibrosis in chronic inflammatory conditions, such as pulmonary fibrosis (45,119) and bone marrow fibrosis (76). The local constitutive production of IL-1 by the conjunctival epithelial cells, could serve for the paracrine stimulation of target cells such as fibroblasts and induce exaggerated production and accumulation of subconjunctival fibrous tissue in patients with trachoma.

In addition, we have detected cytoplasmic expression of the fibrogenic cytokines IL-1α, IL-1β (discussed above), tumor necrosis factor (TNF)-α and platelet-derived growth factor (PDGF) by macrophages in the substantia propria. Our results are consistent with previous studies that murine *C. trachomatis* infection induces TNF-α production (115). TNF-α stimulates fibroblast proliferation and collagen synthesis (26,119). On the other hand, TNF-α is known to restrict intracellular Chlamydial replication (97). PDGF is a potent chemoattractant and mitogen for fibroblasts and smooth muscle cells and a stimulator of collagen synthesis by fibroblasts (34,78,86,114). These cytokines have the potential to influence the remodeling and fibrosis observed in the conjunctiva of patients with trachoma.

ALTERATIONS IN CONJUNCTIVAL COLLAGEN IN THE CONJUNCTIVA OF PATIENTS WITH TRACHOMA

The collagens which are the major proteins of connective tissue, provide the extracellular framework for all multicellular organisms. A subclass of this family comprises collagens I, II, III, V and XI which form banded fibrils. They have thus been called the fibrillar collagens, to distinguish them from other collagens unable to aggregate into these highly-ordered fibrils. Collagens I, II and III are the most abundant and have therefore been far more studied than the quantitatively minor collagens V and XI. The function of type I collagen is to give tissue tensile strength. Type II collagen is principally present in cartilage and type III collagen is associated with tissues and organs that require a motile structural scaffolding such as uterus, arteries, skin, intestines and lung. Type IV collagen is a non-fibrillar collagen which is only found within basement membranes (27,57).

Histochemically, we observed that the conjunctival substantia propria from patients with active trachoma and scarred trachoma contained collagen type V. The extent of deposition of collagen V was markedly increased in scarred trachoma when compared with active trachoma. On the other hand, collagen V was not detected in the normal conjunctiva. New collagen V deposition was noted close to the basement membranes of the conjunctival epithelium, stromal vascular endothelium and accessory lacrimal glands (Fig. 11) (3,5). This is consistent with previous immunolocalization data that demonstrated collagen V localization in the immediate vicinity of basement membranes suggesting an anchoring function for collagen V between basement membranes and stromal matrix (27). Our results are consistent with studies describing accumulation of collagen V in conditions associated with tissue remodeling and new collagen synthesis. Increased type V collagen content has been detected in other diseased tissues such as cardiac hypertrophy (39), neoplasia (10), Crohn's disease (33), atherosclerotic lesions (64), pseudointima of vascular grafts (113) and chronically inflamed gingival tissue (67) suggesting that type V collagen may play an important role in the pathology of these diseases. A number of factors have been shown to induce type V collagen expression including transforming growth factor-beta 1 (69), epidermal growth factor (88), hepatic fibrogenic factor (20), angiotensin II (44) and platelet derived growth factor (69).

We have observed that the conjunctival tissue from patients with active trachoma contained increased amounts of collagen types I, III and IV. Scarred trachoma is characterized by marked increase in basement membrane-collagen IV (Fig. 12). In contrast, collagen types I and III immunoreactivity was markedly decreased when compared with active trachoma (3,5). These observations are well in agreement with the findings reported in wound healing. In the early phase of wound healing the expression of α_1 (I) and α_1 (III) collagen chains is coordinately increased. In later stages the gene expression is rapidly down regulated (70,84). In addition, our findings agree well with immunohistochemical studies of kidney biopsies from patients with chronic renal disease. The severity of interstitial extracellular matrix accumulation was accompanied by significant increase in the deposition of collagen types IV and V. On the other hand, the increase in interstitial abnormalities was not accompanied by significant increase in the deposition of the interstitial collagen types I and III (107). Our results in active trachoma and scarred trachoma are also in agreement with experimental autoimmune uveoretinitis studies. Increased immunoreactivity of collagen types I, III and V was noted at the peak of inflammation. A decrease in immunoreactivity for collagen types I and III but persistant immunoreactivity for collagen type V was observed in later stages (54). Previous histopathologic studies of conjunctival biopsies from patients with cicatricial entropion complicating trachoma showed a thick and compact subepithelial fibrous membrane (8). Our data indicate that the increased deposition of collagen type IV in the epithelial basement membrane and new type V collagen formation in the immediate vicinity of the epithelial basement membrane might contribute to the formation of this subepithelial scar tissue. The additional increase of immunoreactivity for types IV and V collagen around the acini of accessory lacrimal glands might contribute to dysfunction of these glands leading to dry eye disease.

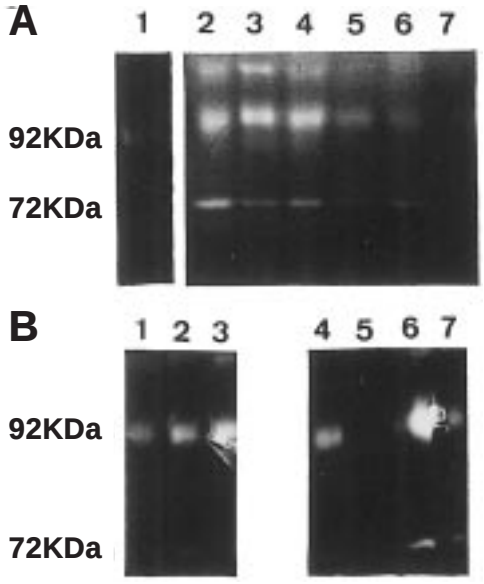
EXPRESSION OF GELATINASE B IN TRACHOMATOUS CONJUNCTIVITIS

Metabolic alterations of extracellular matrix components and collagen metabolism occur in the conjunctival tissue from patients with trachoma (3,5). The matrix metalloproteinases are recognized as key enzymes both for normal extracellular matrix turnover and for the exaggerated extracellular matrix breakdown associated with pathologic conditions, including tumor invasion and metastasis, angiogenesis, inflammatory reactions, wound healing and scar formation (58,72,116). The major members of this family include the following: collagenases, which degrade and denature fibrillar collagen types I, II and III; gelatinases A and B (respectively, the 65-Kd to 75 -Kd matrix metalloproteinase-2 and the 85-Kd to 96-Kd matrix metalloproteinase-9) which cleave denatured

collagens (gelatins), collagen types IV, V, VII and X, elastin and fibronectin; and stromelysins, which degrade proteoglycans, laminin, fibronectin, type IV collagen and the globular domains of other extracellular matrix macromeclules (116). More recently, membrane type matrix metalloproteinase expressed on cell membranes is identified as a fourth category (82). Because of its unique and broad substrate specificity, its involvement in other chronic inflammatory and autoimmune diseases (71) and its distal position in the matrix proteolytic cascade we hypothesized that excessive expression of gelatinase B may play a role in matrix degradation in trachomatous conjunctivitis. To test this hypothesis, we examined conjunctival specimens obtained from patients with active trachoma using immunohistochemistry and gelatin zymography. The findings in trachoma were compared with the findings in the conjunctiva from normal individuals.

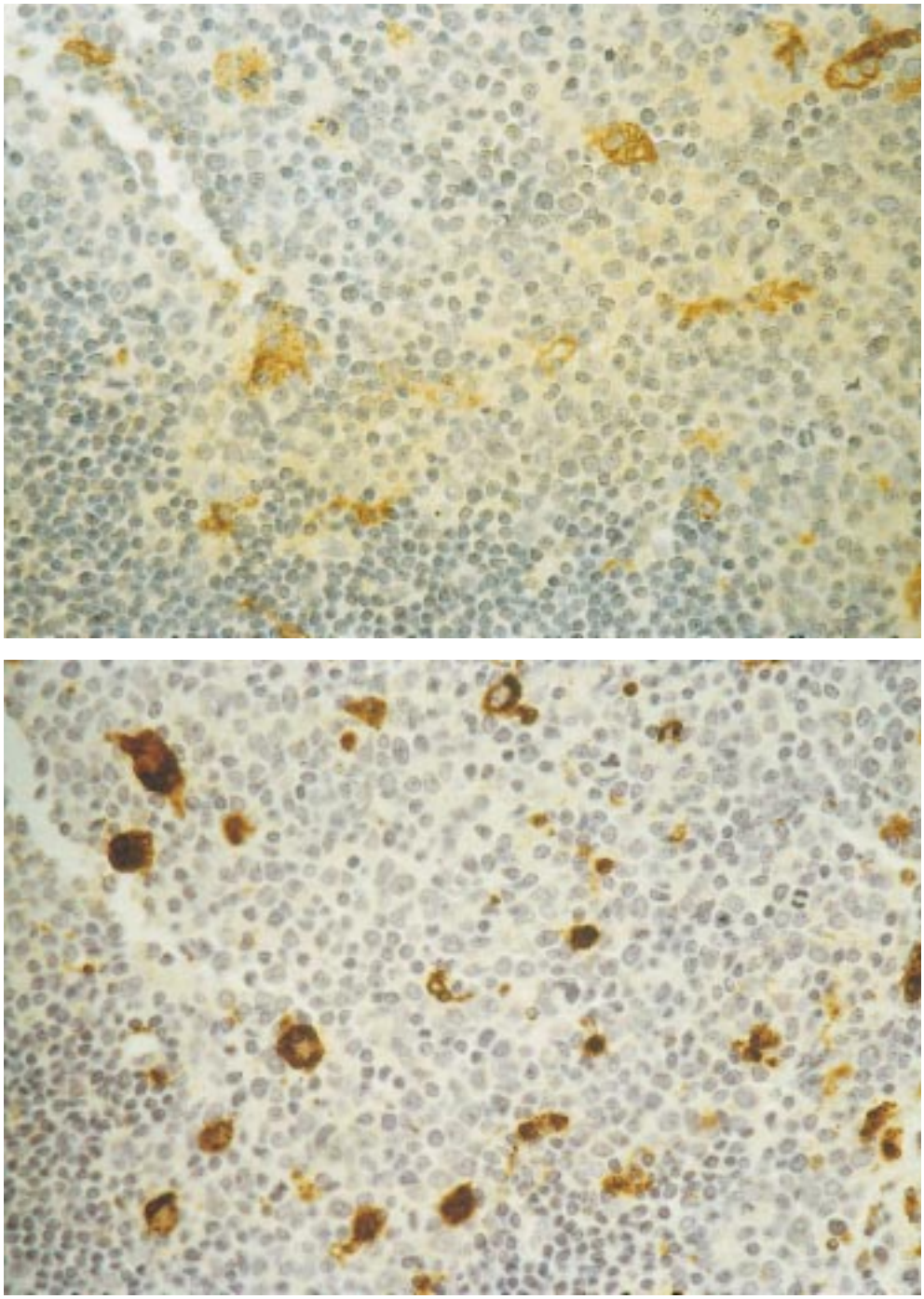
Zymography indicated an increasing activity of gelatinase B in trachoma specimens (Fig. 13). Using immunohistochemistry, we have demonstrated that gelatinase B was specifically localized in macrophages and neutrophils present in the inflammatory infiltrate in trachoma specimens (Fig. 14) (2). Macrophages, monocytes and neutrophils are the main producers of gelatinase B (37,66). Most of the stromal neutrophils were in the intravascular spaces indicating that macrophages were primarily responsible for the production of gelatinase B. Macrophages synthesize and secrete matrix metalloproteinases which are capable of degrading all macromolecular constituents of the extracellular matrix (111). These enzymes appear to function as a proteolytic cascade of which gelatinase B is a downstream element and whose activation is initiated after that of stromelysin (32). The intracellular localization of gelatinase B in macrophages can be interpreted as evidence of active synthesis of this enzyme because macrophages do not store this metalloproteinase (37).

Fig. 13 Gelatin zymography of conjunctival biopsy specimens from normal subjects (A) and from patients with active trachoma (B). The zymographies of these samples show the presence of both gelatinase a (72 KDa) and gelatinase B (92 KDa). (Reproduced with permission from reference 2)



The mechanism by which *C. trachomatis* induces gelatinase B expression by macrophages has not been determined. The effects of *C. trachomatis* on the synthesis and release of this enzyme may be related to the production of cytokines. *C. trachomatis* is an effective inducer of IL-1 and TNF-α production by monocytes (42,79). Whole spleen cells produced TNF-α in vitro in response to murine *C. trachomatis* in a mouse model of pneumonia caused by murine *C. trachomatis* (115). IL-1α and IL-1β mRNA and bioactivity are induced in murine lungs in response to Chlamydial infection (52). In agreement with these studies we have demonstrated upregulated production of IL-1α, IL-1β and TNF-α in the conjunctiva from patients with active trachoma (4). TNF-α and IL-1β selectively up-regulate gelatinase B by macrophages (81,106). As in all gram-negative bacteria, the outermost monolayer of *C. trachomatis* is composed of LPS, a complex glycolipid essential for bacterial survival (15,19). LPS is a potent inducer of the biosynthesis and secretion of gelatinase B in macrophages (80,101,106,111,117). Ingalls and associates (42) demonstrated that the inflammatory cytokine response to *C. trachomatis* infection is mediated primarily through LPS and can be completely blocked by a specific LPS antagonist. Chlamydial LPS induces mononuclear phagocytes to produce TNF-α and IL-1β (42,79). Thus it is possible that gelatinase B production by mononuclear cells may be influenced

Fig. 14
Serial sections immunohistochemical stainings for gelatinase B (top) and macrophage marker (CD68) (bottom). Nearly all macrophages are positive for gelatinase B (x 500). (Reproduced with permission from reference 2)



by these cytokines acting through autocrine pathways. However, Saarialho-Kere and associates (80) have shown that the LPS-mediated effects on gelatinase B production are probably transduced primarily by signaling pathways through the LPS receptor. Although Chlamydial LPS has been shown to elicit secretion of proinflammatory cytokines, Ingalls and associates (42) showed that the potency of Chlamydial LPS was 100-fold less than that elicited by LPS from *Neisseria gonorrhoeae* or from *Salmonella minnesota*. According to these investigators, this relatively weak induction of an acute immune response may partially explain the asymptomatic nature of *Chlamydial* infection.

Gelatinase B degrades denatured collagens (gelatin), collagen types IV, V, VII and X, elastin and fibronectin (65,85). Therefore, expression of gelatinase B in trachoma may contribute to progressive breakdown of conjunctiva by degrading minor constituents of the extracellular matrix. The degradation of fibrillar collagen types I, II and III is initiated by collagenases and completed by gelatinase B. The degradation of collagen types IV and V associated with basement membranes permits the extravasation of macrophages into conjunctival tissue. It has been demonstrated that in vitro migration of T lymphocytes across a basement membrane equivalent is mediated by gelatinase B (48). Macrophage gelatinase B might enhance fibroblast migration into conjunctival epithelium by creating breaks in epithelial basement membrane. Since collagen fragments may be chemotactic for monocytes, degradation of collagen by macrophages could further promote monocyte recruitment (55). In addition, chronic release of specific collagen fragments in the conjunctiva by gelatinase B might contribute to selection of autoreactive T cells and generation of auto-immunity (71).

CONCLUSIONS

On the basis of our data and of other in vitro and in vivo animal studies, the following immunopathogenesis of trachomatous keratoconjunctivitis can be proposed. *Chlamydia trachomatis* replication in the conjunctival and corneal epithelial cells releases a number of pathogenic antigenic proteins including the major outer membrane protein, 60 KD cysteine-rich protein and 57 KD and 75 KD heat-shock proteins. In addition, Chlamydial lipopolysaccharide is released (21,62,68,73,90,94). *C. trachomatis* stimulates B-lymphocyte proliferation and differentiation into plasma cells secreting large quantities of immunoglobulins. This response is enhanced by T-lymphocytes (49). These antibodies are capable of neutralizing *Chlamydiae*, block attachment and internalization and can thereby produce partial immunity (94,112). Stimulation with *C. trachomatis* elementary bodies elicits IL-6 formation by B-lymphocytes (28) which induces the final differentiation of B cells to antibody-producing cells (38). Antigen-presenting cells process and present Chlamydial antigens to CD_4^+ T-lymphocytes in an MHC class II molecule-restricted manner, resulting in helper T-lymphocyte proliferation and activation, and in secretion of several cytokines (90,95) that promote differentiation and clonal expansion of several different subsets of *Chlamydia*-committed effector CD_8^+ cytotoxic T-lymphocytes. The majority of CD_4 T-lymphocytes are belonging to TH1 cells producing IL-2 and IFN-γ (18,89). T-lymphocytes and cell-mediated immunity are essential for the eradication of Chlamydial infections (91,93). T-lymphocyte stimulation by *C. trachomatis* elementary bodies elicits IFN-γ production (28) which has been shown to inhibit the intracellular growth of Chlamydia (97) and to exert cytotoxic activity against cells infected with *C. trachomatis* (17). TNF-α is produced in response to *C. trachomatis* infection (115) and exhibits a synergistic effect with IFN-γ to restrict intracellular growth of *C. trachomatis* (97). CD_8^+ cytotoxic T-lymphocytes contribute to antichlamydial T-cell immunity. CD_8^+ T cells lyse *Chlamydia*-infected cells and recognize a peptide epitope on infected cells in the context of MHC class I molecules. Their ability to resolve Chlamydial infection is correlated with the capacity to elaborate IFN-γ and TNF-α (40,91). On the other hand, stimulation by IFN-γ induces aberrant MHC class II antigens expression by the infected conjunctival and corneal epithelial cells which may lead to perpetuation of the inflammatory immune response, induction of an autoimmune reaction and progression of conjunctival and corneal scarring. T-lymphocytes produce mediators in-

cluding IL-2 and IFN-γ which trigger macrophages to produce fibrogenic cytokines. Furthermore, T-lymphocytes produce transforming growth factor-β which stimulates collagen synthesis (46). Colony-stimulating factors (CSFs) are produced by CD_4^+ cells in response to *C. trachomatis* infection (53). CSFs are cytokines involved in the production, differentiation and activation of phagocytic cells. Macrophages are effector cells in cell-mediated immunity to *C. trachomatis* and inhibit intracellular replication of *C. trachomatis* (56). On the other hand, macrophages play a central role in normal wound healing and pathologic fibrosis by virtue of their ability to release a variety of fibrogenic cytokines (46). Conjunctival macrophage activation in trachoma is suggested by our findings of cytoplasmic expression of IL-1α, IL-1β, TNF-α and PDGF that may be important in the pathogenesis of scarring (4). In addition, active synthesis of gelatinase B by macrophages might be involved in matrix degradation and promotion of conjunctival scarring in trachoma (2). Cell-mediated immunity to ocular infection with *C. trachomatis* may thus be a two-edged sword. Furthermore, the local constitutive production of IL-1 by the conjunctival epithelial cells as shown by our data (4) could stimulate fibroblast proliferation and induce exaggerated production and accumulation of subconjunctival fibrous tissue in patients with trachoma.

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