

Cytokine and Fibrogenic Gene Expression in the Conjunctivas of Subjects from a Gambian Community Where Trachoma Is Endemic

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Received 13 May 2004/Returned for modification 30 June 2004/Accepted 21 August 2004

The role of immunity in blinding trachoma is unclear. Conjunctival gene expression was measured in a population where trachoma is endemic. Proinflammatory (tumor necrosis factor alpha and interleukin-1 β [IL-1 β]), anti-inflammatory (IL-10), and fibrogenic (matrix metalloproteinase 9) gene expression was increased in active trachoma. Markers indicative of T-cell response (gamma interferon, IL-4, IL-12p40, and perforin) were increased when chlamydial infection was present.

Trachoma is the leading infectious cause of blindness (39). Repeated conjunctival infection with *Chlamydia trachomatis* drives a progressive process of scarring, trichiasis, and corneal opacification. While the conjunctival immune response is important in resolving infection, immunopathological events may initiate the scarring process (21). Similar scarring occurs in genital *C. trachomatis* infection, where it leads to infertility and ectopic pregnancy.

Our understanding of which immunological responses promote resolution of human *C. trachomatis* infection and which promote scarring is currently limited. This needs to be improved if a vaccine capable of inducing protection without risk of tissue damage is to be developed. We studied patterns of conjunctival immunological and fibrogenic responses in a community where trachoma is endemic by measuring mRNA expression, using quantitative reverse transcriptase PCR (RT-PCR), for informative cytokine and fibrogenic factors in relationship to disease and infection.

The study was approved by the Gambian Government/Medical Research Council Joint Ethics Committee. All available residents of two Gambian villages were examined for active trachoma (F2/F3 or P3) by an ophthalmologist (13). Swab samples from the left upper tarsal conjunctival surface were collected into RNALater (Ambion Inc., Austin, Tex.) on ice and stored at -20°C .

Total RNA was extracted with the RNeasy minikit (QIAGEN, Crawley, United Kingdom). *C. trachomatis* 16S rRNA expression was quantitated in duplicate by a one-step, real-time RT-PCR using the QuantiTect SYBR Green RT-PCR kit (QIAGEN) with previously described primers (Table 1) (22) on an ABI 5700 sequence detection system (Applied Biosystems, Warrington, United Kingdom). Human RNA was reverse transcribed with the Omniscript RT kit (QIAGEN) and oligo-dT₁₅ primers. The expression of human genes (Table 1)

was quantitated in duplicate by two-step, real-time RT-PCR with the QuantiTect SYBR Green PCR kit (QIAGEN). A standard calibration curve was generated for each run of the assay. Standards were produced by serial 10-fold dilutions of known amounts of target DNA in ultrapure water with 2 ng of herring sperm DNA/ μl . Results are presented as a ratio to the expression of hypoxanthine phosphoribosyl transferase 1 (HPRT-1) in the same sample.

In all, 248 subjects participated (79% of the total population). The median age was 12 years, all were of the Wolof ethnic group, and 59% were female. Clinically active trachoma was diagnosed in 42 subjects (16.9%), and infection (*C. trachomatis* 16S rRNA expression) was detected in 17 (6.8%) subjects, including 14 with active trachoma.

The mRNA expression of various cytokines and fibrogenic factors (Table 1) was quantitated in all 248 samples. Insufficient sample volume precluded measurement of matrix metalloproteinase 9 (MMP-9) and type I collagen in 2 and 42 specimens, respectively. Transforming growth factor β 1 (TGF- β 1) was assayed in 82 samples and found to be undetectable or present at only very low levels. Data were analyzed for three subject groups by using STATA 7 and Genstat 6.1: group 1, 203 noninfected, clinically normal; group 2, 17 infected; and group 3, 28 noninfected but with active disease. The geometric mean and median values of the various targets by these subdivisions are shown in Table 2.

Comparisons across disease and infection states were made with a linear mixed model (after \log_{10} transformation of the ratios), adjusted for village or compound clustering (random effect) and for gender and age. Targets for which the disease or infection state had a significant effect (at $P = 0.05$) were compared between groups by using the Wald test (Table 3), adjusted for multiplicity by the Holm method (giving a critical significance level of $P = 0.0049$).

Active trachoma without *C. trachomatis* infection was associated with increased expression of the cytokines tumor necrosis factor alpha (TNF- α), interleukin-1 β (IL-1 β), and IL-10; subjects infected with *C. trachomatis* additionally had increased expression of gamma interferon (IFN- γ), IL-12p40, and per-

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TABLE 1. Sequences of the primers used in this study^a

Target	Sequences
<i>C. trachomatis</i> 16S rRNA	GGAGAAAAGGAATTCACG TCCACATCAAGTATGCATCG
HPRT	GGCAGTATAATCCAAAGATGGTCAA GTCTGGCTTATATCCAACACTTCGT
IFN- γ	ATTCGGTAACTGACTTGAATGTCC CTCTTCGACCTCGAAACAGC
TNF- α	TGCTTGTTCCTCAGCCTCTT GGTTTGTCTACAACATGGCTAC
IL-1 β	ATTGCTCAAGTGTCTGAAGC GTAGTGGTGGTCGGAGATT
IL-2	AATGGAATTAATAACAAGAATCCC TGTTTCAGATCCCTTTAGTTCAG
IL-4	CGAGTTGACCGTAACAGACAT CGTCTTTAGCCTTTCCAAGAAG
IL-10	TGAGAACCAAGACCCAGACA TCATGGCTTTGTAGATGCCT
IL-12p40	CTGCAGTTAGGTTCTGATCCAG GGCAACTTGAGAGCTGGAAA
IL-12p35	TTATGATGGCCCTGTGCC TCAAGGGAGGATTTTTGTGG
Perforin	GCTTCTACAGTTTCCATGTG GTGCCGTAGTTGGAGATAAG
TGF- β 1	ACGCAGTACAGCAAGGTCTCG GGGCACGGTGTCTTAAATA
TGF- β 2	GCTTTAGAAATGTGCAGGATAATTG TAAGCTCAGGACCCTGCTGT
MMP-1	GCTAACAAATACTGGAGGTATGATG TTTTCTGCAGTTGAACCAG
MMP-9	GTCTTCCAGTACCGAGAGAAAAG GTTTGTATCCGGCAAACCTGG
Type 1 collagen (COL1A1)	TGGTGCTAAGGGTGATGCTG CCATCTTTGCCAGGAGAGC

^a The top sequence is the forward primer of each pair.

Table 3. Comparisons between results for different disease and infection states for various targets by the Wald test (*P* value)

Target	<i>P</i> value for:		
	Uninfected, clinically normal vs infected	Uninfected, clinically normal vs uninfected, active trachoma	Infected vs uninfected, active trachoma
IFN- γ	<0.0001	0.07	0.0007
TNF- α	<0.0001	0.0001	0.02
IL-1 β	<0.0001	<0.0001	0.79
IL-10	<0.0001	<0.0001	0.02
IL-12p40	0.0015	0.92	0.006
Perforin	<0.0001	0.05	<0.0001
MMP9	0.0026	0.0021	0.6
Collagen I/MMP-1	0.023	0.28	0.17

forin (Table 2). IL-2 showed a nonsignificant trend towards increased expression in subjects with infection. IL-4 expression was discrete. Where present, it was found at low levels within a narrow range. IL-4 was detected in 99 of 203 (49%) noninfected, clinically normal subjects, 15 of 17 (88%) infected subjects, and 18 of 28 (64%) noninfected, active disease subjects. Infected individuals had significantly more expression of IL-4 than the normal group (odds ratio, 7.88; 95% confidence interval [CI], 1.76 to 35.3; *P* = 0.002, Fisher's exact test). The IL-12p35 mRNA component of the IL-12 heterodimer (IL-12p75) is known to be constitutively expressed (9) and accordingly did not vary with disease or infection.

The fibrogenic factors TGF- β 2, MMP-1, and type I collagen were readily detected in all samples but did not vary with disease or infection state (Table 2). Active disease and chlamydial infection were associated, however, with significantly increased expression of MMP-9 and a small increase in the type I collagen/MMP-1 ratio (Table 2). MMP-9 expression also increased with increasing severity of inflammation (Table 4).

Canonical variate analysis was used to identify linear combinations of these target ratios that best discriminate the three subgroups by maximizing between-group-to-within-group variability. The 95% confidence regions for the group canonical means demonstrate clear separation between the groups (Fig. 1).

TABLE 2. Geometric mean and median values of the ratio of the expression of various targets to HPRT by clinical and infection status

Target	Uninfected, clinically normal (<i>n</i> = 203)			Infected (<i>n</i> = 17)			Uninfected, active trachoma (<i>n</i> = 28)		
	Mean	95% CI	Median	Mean	95% CI	Median	Mean	95% CI	Median
IFN- γ	0.48	0.43–0.52	0.48	1.48	1.01–2.16	1.16	0.71	0.53–0.94	0.73
TNF- α	0.13	0.11–0.16	0.12	1.10	0.66–1.83	0.66	0.58	0.38–0.88	0.59
IL-1 β	0.11	0.09–0.14	0.09	0.91	0.52–1.58	1.09	0.89	0.48–1.65	0.68
IL-2	0.003	0.002–0.003	0.003	0.007	0.004–0.011	0.005	0.002	0.002–0.004	0.003
IL-4			0			0.0003			0.0002
IL-10	0.11	0.09–0.12	0.09	0.88	0.48–1.63	1.00	0.43	0.27–0.69	1.00
IL-12p35	0.03	0.02–0.03	0.028	0.03	0.02–0.03	0.025	0.02	0.02–0.03	0.021
IL-12p40	0.009	0.008–0.011	0.011	0.025	0.016–0.04	0.025	0.010	0.007–0.015	0.012
Perforin	0.07	0.06–0.08	0.07	0.24	0.15–0.37	0.27	0.10	0.08–0.14	0.09
TGF- β 2	0.04	0.03–0.04	0.04	0.04	0.03–0.06	0.04	0.03	0.02–0.04	0.03
MMP-1	1.12	0.98–1.30	1.25	0.59	0.32–1.08	0.59		0.94	0.66–1.33
MMP-9	0.26	0.20–0.33	0.33	0.95	0.33–2.73	1.73	0.97	0.53–1.77	1.14
Collagen I	0.12	0.11–0.14	0.13	0.15	0.10–0.24	0.14	0.15	0.10–0.22	0.16
Collagen I/MMP1	0.10	0.09–0.13	0.10	0.29	0.19–0.46	0.32	0.16	0.09–0.27	0.15

TABLE 4. Geometric mean and median values of MMP-9/HPRT ratio by the degree of papillary inflammation

Papillary inflammation score ^a	Geometric mean	95% CI	Median
P0	0.28	0.22–0.36	0.37
P1	0.20	0.07–0.59	0.18
P2	1.43	0.68–2.99	1.71
P3	3.01	1.54–5.88	2.74

^a See reference 13 for details.

The critical event in the pathogenesis of blinding trachoma is the development of chronic conjunctival inflammation, triggered by *C. trachomatis* infection. Scarring and trichiasis develop in a minority of individuals, suggesting that variations in susceptibility are important. Severe inflammatory trachoma is associated with increased risk of cicatricial complications later in life (14, 40).

These data indicate that active trachoma is associated with increased expression of the proinflammatory cytokines IL-1 β and TNF- α . Other studies have found similar associations (3, 8, 10). IL-1 β and TNF- α induce MMP activation, collagen production, and fibroblast activation (23, 38, 46), and their prolonged expression is associated with several chronic inflammatory and fibrotic conditions such as rheumatoid arthritis and pulmonary fibrosis (20). These findings support the view that these are important mediators of inflammation in trachoma,

whose continued presence following resolution of infection may contribute to the development of scarring.

We found increased IL-10 expression associated with active trachoma. In contrast, a previous study in trachomatous subjects failed to detect IL-10 (8). The effect of IL-10 in trachoma is unclear; it may limit immune-mediated tissue damage through its anti-inflammatory and immunoregulatory effects (27); however, it could also curtail the effector arm of the cell-mediated immune (CMI) response, allowing *C. trachomatis* to persist (35, 47). We show elsewhere that polymorphism at the IL-10 locus is associated with risk of scarring, and increased IL-10 mRNA from *C. trachomatis* antigen stimulated peripheral blood mononuclear cells in subjects with trachomatous scarring (16, 26).

C. trachomatis infection was associated with a cytokine response suggestive of activated CMI with significantly increased expression of IFN- γ , IL-4, IL-12p40, and perforin mRNA. IFN- γ is important in the control of chlamydial infections, through several well-described mechanisms (34). In mice, impairment of IFN- γ resulted in prolonged and disseminated infection (12, 19, 32). Peripheral blood mononuclear cells from a population where trachoma is endemic proliferate and produce IFN- γ in response to chlamydial antigens; these responses were weaker in subjects with trachomatous scarring, suggesting that a poor CMI response might be associated with fibrosis through more prolonged infection episodes (16, 17). Increased IL-2 expression with infection suggests T-cell proliferation and is consistent with a previous study of conjunctival

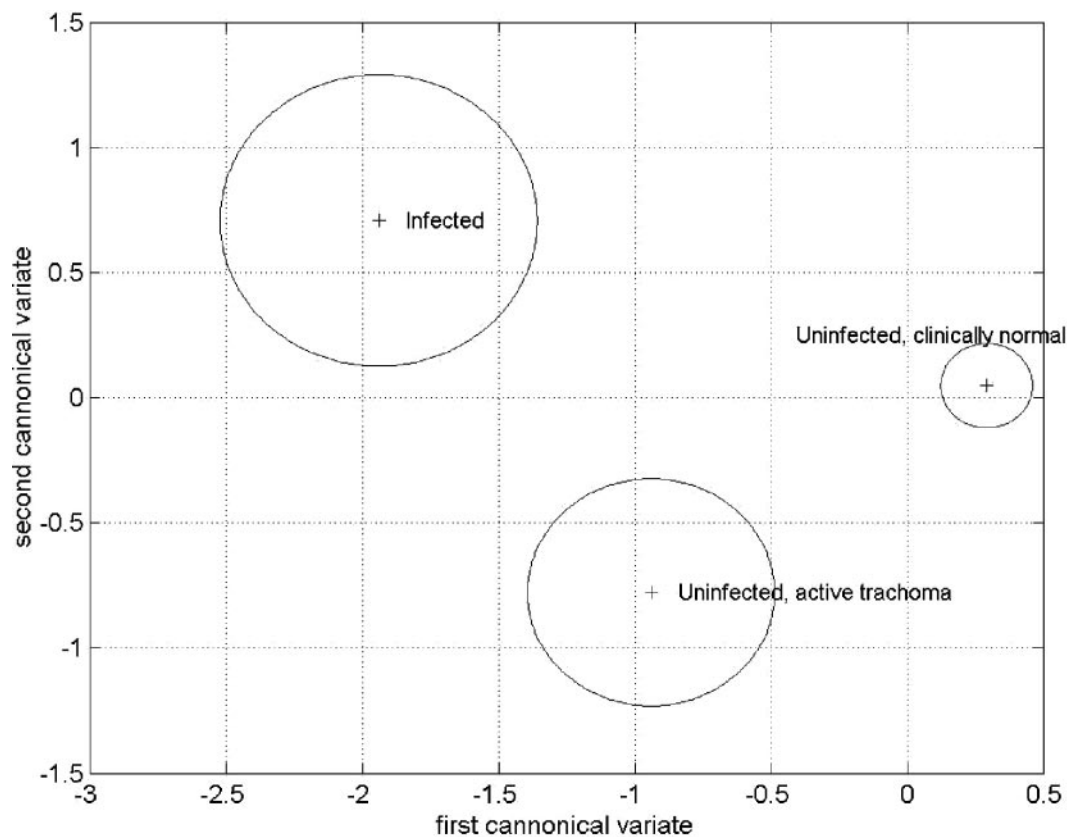


FIG. 1. Canonical means for different infection and disease states (+), with 95% confidence regions (circles).

gene expression in trachoma (8). IL-12 from dendritic cells and macrophages links the innate and acquired immune responses, driving the proliferation of IFN- γ -producing TH1 cells. In mice, neutralization of IL-12 is associated with reduced levels of IFN- γ and prolonged *C. trachomatis* infections (29).

The finding of increased IL-4 expression with infection, albeit at low levels, may indicate the presence of TH0, TH2, or other cells, such as natural killer and mast cells. Although resolution of infection probably requires a CMI response dominated by IFN- γ , this may be counterbalanced by a number of factors, including IL-4. These mixed findings are typical of the blend of TH responses frequently observed in human infectious diseases, in contrast to a more polarized response observed in mice. Animal models do not suggest that IL-4 contributes to the resolution of infection (29, 42), although it is expressed in infected tissue (24).

The expression of perforin was used as a marker for cytotoxic T-cell (CTL) activity. CTLs may have an antichlamydial role by targeting infected conjunctival cells and may help contain conjunctival inflammation through leukocyte apoptosis. In mice, CTLs are found in infected tissue (24) but are not necessary for resolution of infection (25, 30). Conjunctival biopsies from children with active trachoma show both CD4⁺ and CD8⁺ cells infiltrating the substantia propria (3). Specific antichlamydial CTL responses were found in peripheral blood from adults without scarring and children recovering from active disease (18).

Understanding the fibrogenic process may be the key to unravelling the pathogenesis of trachoma. MMPs are proteolytic enzymes that regulate the extracellular matrix (ECM) and are implicated in fibrotic disease processes (45). Active trachoma, particularly severe inflammation (inflammatory trachoma), and *C. trachomatis* infection were associated with increased expression of MMP-9. This enzyme may be central to the pathogenesis of trachomatous scarring. Increased MMP-9 expression and functional activity have been demonstrated in conjunctival biopsies from children with active trachoma (15). Studies indicate that *C. trachomatis* infection is associated with increased MMP-9 activity (6, 31). MMP-9 is activated by proinflammatory cytokines (23, 36) and may in turn perpetuate the inflammation by proteolytic activation of IL-1 β (37). MMP-9 degrades the ECM, rendering the conjunctiva more plastic, facilitating the migration of cells, including fibroblasts. MMP-9 can activate TGF- β (5), which promotes scar tissue deposition (7, 11). TGF- β can increase MMP expression, perpetuating this process (41). Interestingly, postoperative inhibition of MMP activity reduces conjunctival scarring (44).

There are three human isoforms of TGF- β , of which TGF- β 2 is predominant in conjunctival tissue (28). We found TGF- β 2, but not TGF- β 1, was readily detectable in all subjects but did not vary with infection or disease. The regulation of TGF- β is largely posttranscriptional; therefore, this finding does not exclude a role for TGF- β in the pathogenesis of scarring trachoma. In vitro and in vivo studies suggest that TGF- β activity increases in *C. trachomatis* infection (33, 43).

In advanced trachomatous scarring, a thick band of fibrotic tissue composed mostly of type V collagen replaces the normal stromal tissue (2, 4). In biopsies from children with active trachoma, there is an increase in type I and type III collagen between epithelial cells and in the stroma (1). We found the

expression of type I collagen did not vary with active disease or infection; however, the type I collagen/MMP-1 expression ratio was higher in both the presence of infection and disease, which could lead to an increase in the amount of collagen deposited in the conjunctiva.

This study demonstrated that active trachoma was characterized by increased expression of TNF- α , IL-1 β , IL-10, and MMP-9. In addition, *C. trachomatis* infection was also associated with cytokines characteristic of a CMI response: IFN- γ , IL-4, IL-12p40, and perforin. Increased expression of MMP-9 in both disease and infection is of particular interest and may provide a mechanism whereby inflammation and ECM breakdown self-perpetuate in the absence of infection and may therefore have an important role in the development of trachomatous scarring.

We thank the residents of the villages participating in this study for their good-humored cooperation.

This work was supported by grants from the Medical Research Council and the Wellcome Trust/Burroughs Wellcome Fund.

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