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T helper type-1 (Th1)/Th2 profiles of peripheral blood mononuclear cells (PBMC); responses to antigens of *Chlamydia trachomatis* in subjects with severe trachomatous scarring

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SUMMARY

Increased stimulation of Th2 cytokines may contribute to the development of persistent ocular chlamydial infection, resulting in the blinding pathological changes of trachoma. Proliferation and cytokine production profiles of PBMC in response to stimulation with antigens of *Chlamydia trachomatis* were compared in 30 patients with severe conjunctival scarring due to trachoma and in 30 age-, sex- and location-matched controls. Interferon-gamma (IFN-γ) and IL-4 were detected at the single-cell level by ELISPOT assay. Transcription of the genes encoding IFN-γ, IL-4 and IL-10 was detected in mRNA isolated from parallel cultures of PBMC using reverse transcriptase-polymerase chain reaction (RT-PCR). Incubation with the chlamydial heat shock protein (hsp)60 resulted in increased numbers of IL-4-producing cells in PBMC isolated from patients with scarring disease and increased secretion of IFN-γ from PBMC of control subjects. Incubation with the chlamydial major outer membrane protein (MOMP) increased the number of IFN-γ-producing cells in the control group only. Messenger RNA encoding IL-4 was only detected in PBMC of patients with scarring disease after *in vitro* stimulation with chlamydial antigens, but IFN-γ mRNA and IL-10 mRNA were also more frequently detected in this group. Thirty-eight subjects were HLA-DRB1 and -DQB1 typed. Associations were observed between certain HLA class II alleles and cellular immune responses to chlamydial antigens. No HLA associations were found with clinical status, and overall we found no evidence of strong associations and the type of immune response. These data are consistent with a role for Th2 cells and cytokines in the pathogenesis of trachomatous scarring.

Keywords cell-mediated immunity *Chlamydia trachomatis* cytokines

INTRODUCTION

Chlamydia trachomatis is an obligate intracellular Gram- negative bacterium responsible for a range of diseases of mucosal epithelia. It is the most common infectious cause of blindness and the most prevalent bacterial sexually transmitted pathogen [1,2]. Since both ocular and genital infections are often asymptomatic in their early stages, control at present would depend on screening or, in the case of trachoma, mass antibiotic treatment. Neither of these options are logistically or economically feasible in most populations. A vaccine against *C. trachomatis* infection is therefore an important priority.

Early studies in primate models suggested that immunity to ocular chlamydial infection was dependent on the presence of serotype-specific antibody in local secretions [3]. More recent evidence suggests that in addition to local antibody responses, cell-mediated immune (CMI) responses play a major role in the clearance and resolution of chlamydial infection. In murine models, *Chlamydia*-specific T cell lines and clones can transfer protection, which is mediated by the production of interferon-gamma (IFN-γ) [4,5]. T cells of both the $CD4^+$ and $CD8^+$ subsets can adoptively transfer protection [6–8]. However, as yet few studies have examined both qualitative and quantitative aspects of the CMI response in human populations exposed to chlamydial ocular or genital infection.

We have previously demonstrated that proliferative responses of PBMC to chlamydial antigens are depressed in subjects with trachomatous conjunctival scarring and in children with persistent active (inflammatory) trachoma compared with the responses of PBMC from appropriate controls [9,10]. Inability to clear ocular infection in spite of high specific antibody levels in serum and

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ocular secretions is presumably associated with an inappropriate cellular immune response. One likely explanation of the dichotomy of this response is an imbalance in the Th subsets which respond, similar to that proposed for other chronic intracellular infections such as leprosy [11] and leishmaniasis [12].

We studied *in vitro* proliferative and cytokine responses of PBMC to chlamydial antigens in 30 subjects with conjunctival scarring due to trachoma, and 30 age-, sex- and location-matched controls. The cytokines studied include those known to be important in immunity to chlamydial infection (IFN-γ) and are representative of the Th1 (IFN-γ) and Th2 (IL-4, IL-10) subsets. Cytokine production in response to chlamydial antigens was assessed by assay of IFN-γ in culture supernatant, by the enumeration of PBMC secreting IFN-γ and IL-4, and by the detection of mRNA for IFN-γ, IL-4 and IL-10 using reverse transcriptase-polymerase chain reaction (RT-PCR). Within a subset of subjects we have examined associations between polymorphism of HLA class II genes (DRB1 and DQB1) and these immune responses.

We found that stimulation with the chlamydial heat shock protein (hsp)60 resulted in increased numbers of IL-4- producing cells in PBMC isolated from patients with scarring disease. In addition, IL-4 mRNA was detected exclusively in PBMC of patients with scarring disease after *in vitro* stimulation with chlamydial antigens. Stimulation of PBMC from the control group with hsp60 increased secretion of IFN-γ, whilst stimulation with chlamydial major outer membrane protein (MOMP) resulted in increased levels of IFN-γ-producing cells. These data suggest a role for Th2 cells and cytokines in the pathogenesis of trachomatous scarring.

MATERIALS AND METHODS

Subjects

Study participants were consenting adults and adolescents from the villages of Jali and Berending in The Gambia. Clinical signs were assessed and trachoma graded according to the criteria of Dawson *et al.* [1]. Thirty individuals were selected with obvious conjunctival scarring but without features of active disease. Control subjects, without evidence of conjunctival scarring or active disease, were pair matched for age and sex and chosen from the same or adjacent households as the scarred subjects. The mean age of these individuals was 46·7 years (range 12–83 years). In a minority of pairs ($n = 5$) the age difference between the pairs was >10 years but the age distribution was similar between cases and controls. A sample size of 30, each chosen with a matched pair, gave a power of 79% to detect a five-fold difference between cases and controls with 95% confidence.

Detection of Chlamydia

Swabs were taken from the subtarsal conjunctiva for the detection of chlamydial lipopolysaccharide (LPS) by ELISA (Dako Diagnostics Ltd, Ely, UK) according to the manufacturer's instructions.

Antigens

Chlamydia trachomatis serotype A (AS-1 strain; prepared from an isolate from a Jali trachoma patient) elementary bodies (EB) were prepared and purified as previously described [13] and the total protein estimated by the method of Lowry. Serotype A MOMP was supplied as a β-galactosidase (β-gal) fusion protein which contained the carboxy-terminal 371 amino acids of MOMP and seven residues from the β-gal protein. Affinity-purified *C. trachomatis* serovar A hsp60 was used as described previously [13]. Details of the production, expression and purification of these antigens have been published by others [14,15]. Purified protein derivative (PPD) of *Mycobacterium tuberculosis* (Statenserum Institute, Copenhagen, Denmark) was used as a control T cell recall antigen. Phytohaemagglutinin (PHA-P) was used as T cell mitogen (Difco Labs, Detroit, MI).

Lymphocyte cultures

Venous blood (20 ml) was collected into 200 units of preservative-free heparin (Monoparin; John, Bell & Croyden, London, UK) and PBMC isolated by centrifugation over Lymphoprep (Nycomed, Sheldon, Birmingham, UK) [13]. PBMC were resuspended at 10^6/ml in RPMI 1640 supplemented with 5% human AB serum, 2 mM L-glutamine, 10 μg/ml gentamicin sulphate. Aliquots (0·2 ml) were dispensed into 96-well U-bottomed microtitre plates (Nunclon, Roskilde, Denmark) which contained either an antigen (10 μg/ml), PHA (1 : 1000 (v/v)) or medium alone (unstimulated controls). Parallel cultures were established for lymphocyte proliferation, ELISPOT assay, RNA extraction and IFN-γ production, detailed as follows.

Lymphocyte proliferation assays. Stimulation was assessed as previously described [13]. Each antigen was assayed in triplicate, but for negative controls eight replicate wells received cells and culture medium alone. PBMC were cultured for 7 days with addition of 1 μCi/well ^{3}H-thymidine for the last 18 h of culture. After harvesting, cellular incorporation of ^{3}H-thymidine was measured by liquid scintillation counting (LKB-Wallac 1205 Beta plate; Milton Keynes, UK) and the results were expressed as stimulation index (SI), equivalent to geometric mean ct/min (test) divided by the geometric mean ct/min (control).

ELISPOT assay. IL-4- and IFN-γ-producing cells were enumerated using a modification of an ELISPOT assay described by Surcel *et al.* [16]. Briefly, 96-well nitrocellulose microfilteration plates (MAHA S45 10; Millipore, Watford, UK) were coated with 100 μl of a MoAb for IL-4 (MabTech AB, Stockholm, Sweden) or IFN-γ (Quadratech, Epsom, UK) at a concentration of 15 μg/ml in sterile PBS and incubated overnight at 4°C. Unbound antibodies were removed by washing in sterile PBS. PBMC which had been cultured for 24 h as described above were transferred to the antibody-coated wells (1×10^5/well for IFN-γ; 2×10^5/well for IL-4) and incubated for a further 24 h in a humidified 95% air/5% CO_2 atmosphere at 37°C. After incubation cells were removed by washing $\times$ 3 with PBS–0·05% Tween 20 (PBS–T20), each wash consisting of a 5-min soak in wash buffer. Biotin-conjugated monoclonal anti-IL-4 or anti-IFN-γ (100 μl), with differing epitopic specificities to those used to coat the wells, were added to each of the appropriate wells at a concentration of 1 μg/ml. The plates were incubated for 1 h at 37°C before excess antibody was removed by washing as before. Bound second layer antibody was detected by incubation of each well with 100 μl solution of streptavidin-alkaline phosphatase (MabTech AB). Unbound conjugate was removed by washing with PBS–T20. Finally, 100 μl of alkaline phosphatase substrate solution (BioRad, Hercules, CA) were added to each well and incubated until spots appeared in the wells. The reaction was stopped by thorough washing in distilled water. Plates were allowed to dry and the number of spots scored using a dissection microscope (Nikon SMZ, Tokyo, Japan).

RT-PCR for cytokine mRNA. After 48 h of *in vitro* stimulation, total RNA was extracted from the parallel cultures of PBMC according to the method of Gough [17]. Each sample consisted

of 2×10^6 PBMC from which RNA was extracted. cDNA was generated by using Moloney Murine Leukaemia Virus Reverse Transcriptase (MMLV-RT; Life Technologies, Paisley, UK). Subsequently, cDNA was subjected to 30 cycles of PCR amplification in a total volume of 50 μl in the presence of the appropriate oligonucleotide pairs. Sequences for β-actin, IFN-γ, IL-4 and IL-10 were derived from published sequences and supplied as kits (Clontech Labs, Inc., Palo Alto, CA). Each cycle of amplification included 1 min of denaturation at 94°C, 2 min of annealing at 60°C, and 3 min of extension at 72°C. Finally the reaction was completed with a single 7-min extension at 72°C. The size of the PCR products was determined by electrophoresis through 1·5% agarose gels in the presence of ethidium bromide and the bands were visualized and photographed under UV transillumination.

Quantification of IFN-γ in tissue culture supernatants. Cell-free supernatant was collected from the parallel cultures of PBMC which were prepared for RNA extraction. Supernatant was collected immediately before isolation of RNA. IFN-γ was quantified as pg/ml by modification of a two-site ELISA described previously [10]. The fol-lowing modifications were incorporated. First, 100 μl of standard or tissue culture supernatant samples were incubated overnight at room temperature in a moist chamber. Wells were washed $\times 3$ in PBS–T20 at this step and between all subsequent steps. Second, bound rabbit polyclonal anti-human IFN-γ (supplied by Dr H. Dockrell, London School of Hygiene and Tropical Medicine) used to capture IFN-γ was detected by incubation with a 1:250 dilution of a biotin-conjugated mouse anti-rabbit MoAb (Sigma, Poole, UK) for 1 h. Finally, a 1:500 dilution of streptavidin-biotinylated horseradish peroxidase complex (Amersham International, Aylesbury, UK) was added for 30 min at 37°C before the addition of 3,3′,5,5′-tetramethylbenzidine (TMB; Kirkegaard & Perry Labs. Inc, Gaithersburg, MD) and absorbance measured at 630 nm (MR5000, Dynatech, Billinghurst, UK). IFN-γ in pg/ml was determined by reference to standard curves prepared with human recombinant IFN-γ (Genzyme Diagnostics, Cambridge, MA). The sensitivity of this assay was $\approx$ 15 pg/ml.

HLA typing

The 38 subjects in whom immune responses were studied were a subset of a larger case control study designed to examine genetic risk factors for severe scarring trachoma, the results of which will be published elsewhere. HLA-DRB1 and -DQB1 genotypes were determined by PCR amplification from genomic DNA and probing with panels of sequence-specific oligonucleotides (SSO), 20 SSO probes for DRB1 and 15 for DQB1. Methodology, including oligonucleotide PCR primer pairs and SSO probes, followed standard methods [18].

Statistical analysis

In order to preserve the pair-matching of samples in the analysis the RT-PCR data were analysed by McNemar's χ^2 test, with continuity correction. This test is based on the number of discordant case control matched pairs. When the sum of the discordant pairs was <10, the P value from Fisher's exact test was reported. Results from the ELISA test for chlamydial antigen were analysed in a 2×2 contingency table and the P value calculated by Fisher's exact test. All further tests of association and significance on matched pairs (i.e. ELISPOT frequencies, IFN-γ levels in supernatants, SIs and geometric mean ct/min values) were performed by $\log_{10}$ transformation of the data and applying Students t-test in line with recommendations made by Bennett & Riley [19]. For tests of significance between HLA types and the different measures of CMI we avoided dividing the groups into responders and non-responders since there was no *a priori* justification. These sample means were then compared using an independent t-test of the mean of $\log_{10}$ transformed data.

Ethical approval

This study was approved by the Joint Scientific and Ethical Committee of the MRC Laboratories, Fajara, and the Government of the Gambia. Informed consent was obtained from all study participants or their parents/guardians.

RESULTS

Lymphocyte proliferation and IFN-γ production in response to C. trachomatis

The proliferative response of PBMC to *C. trachomatis* serovar A elementary bodies and two recombinant antigens (serovar A MOMP and hsp60) of *C. trachomatis* were studied in 30 case control matched pairs. Figure 1 shows the proliferative responses ($\log_{10}$ SI) measured on day 7. No differences in proliferation could be detected when cases were compared with controls ($P > 0{\cdot}05$ in all tests). Although responses to recombinant antigens were low, the responses were significantly above those observed in unstimulated cells when the sample was considered as a whole ($n = 60$) or separately as cases ($n = 30$) and controls ($n = 30$) ($P < 0{\cdot}001$ in each case).

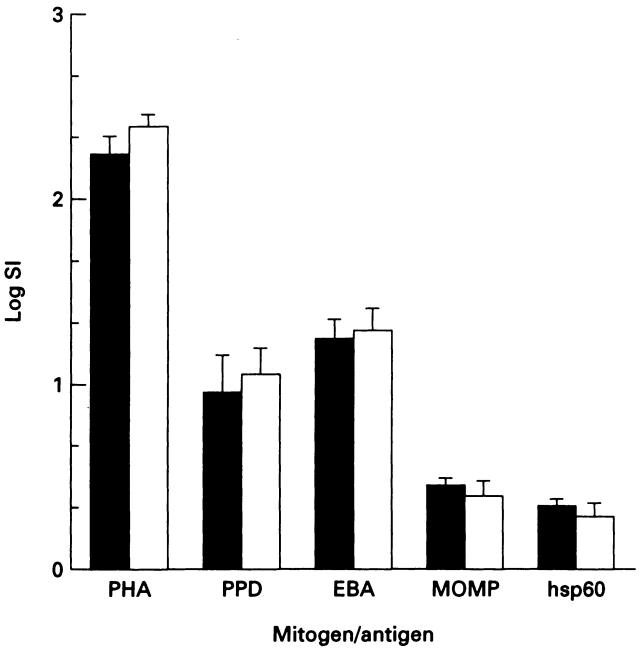

Fig. 1. Proliferative responses $\log_{10}$ stimulation index (SI) of PBMC to phytohaemagglutinin (PHA), purified protein derivative (PPD), serotype A elementary bodies (EB), recombinant serotype A major outer membrane protein (MOMP), and serotype A heat shock protein 60 (hsp60). Geometric mean ct/min $\pm$ s.e.m. for unstimulated cells (medium alone + PBMC); scarring subjects (■, $n = 30$); 211 ± 22, control subjects (□, $n = 30$); 197 ± 20. Difference not significant.

Table 1. Production of IFN-γ by peripheral blood mononuclear cells (PBMC). IFN-γ levels (pg/ml) in supernatants collected after 48 h of culture (mean level with 95% confidence interval (CI) in parentheses) in response to antigens and mitogens

	Mean IFN-γ (pg/ml)	
	Patients $n = 30$	Controls $n = 30$
PHA	8487 (5757–12512)	8400 (5828–12108)
PPD	116 (88–153)	75 (58–96)
EB	407 (214–772)	258 (157–423)
MOMP	44 (29–68)	40 (32–51)
hsp60	27 (23–31)	35 (26–49)
Unstim.	26 (22–39)	25 (22–29)

PHA, Phytohaemagglutinin; PPD, purified protein derivative; EB, serotype A elementary bodies; MOMP, recombinant serotype A major outer membrane protein; hsp60, serotype A heat shock protein 60; unstim., unstimulated cells (medium alone + PBMC).

Table 1 shows IFN-γ levels in tissue culture supernatants collected 48 h after stimulation. Incubation of cells with hsp60 failed to increase IFN-γ secretion above background in the patient ($t = 0{\cdot}56$, d.f. $= 29$, $P = 0{\cdot}58$), but not the control group ($t = 2{\cdot}34$, d.f. $= 29$, $P = 0{\cdot}027$). All other antigens significantly increased IFN-γ levels above background in both groups ($P < 0{\cdot}005$). No significant differences between patient and control matched pairs were detected in the levels of IFN-γ secreted in response to any antigen.

Chlamydial LPS was detected in ocular swab samples from three (10%) of 30 patients with scarring trachoma and in two (6%) of 30 controls (difference not significant).

Frequency of IFN-γ-producing cells

The results presented in Table 2 show the frequencies of IFN-γ-producing cells in response to the antigens and mitogens studied. Significant increases in the frequency of IFN-γ- producing cells were seen in both patients and controls in response to PHA ($P < 0{\cdot}001$) and to whole chlamydial EBs ($P < 0{\cdot}002$) compared with unstimulated cells. Stimulation with MOMP significantly increased the number of IFN-γ-producing cells in the control but not the patient group (controls $t = 2{\cdot}77$, d.f. $= 29$, $P = 0{\cdot}01$; patients $t = 0{\cdot}59$, d.f. $= 24$, $P = 0{\cdot}558$). Stimulation with hsp60 failed to increase significantly the numbers of PBMC producing IFN-γ in either group (patients $t = 1{\cdot}7$, d.f. $= 28$, $P = 0{\cdot}101$; controls $t = 0{\cdot}88$, d.f. $= 24$, $P = 0{\cdot}385$) above that of unstimulated cells.

Frequency of IL-4-producing cells

As detailed in Table 2, PHA stimulation significantly increased the number of IL-4-producing PBMC above background in both patients and controls ($P < 0{\cdot}01$). In response to hsp60, the number of IL-4-producing cells was significantly increased above background in the patient group only (patients $t = 2{\cdot}32$, d.f. $= 23$, $P = 0{\cdot}029$; controls $t = 1{\cdot}93$, d.f. $= 26$, $P = 0{\cdot}067$). All other responses to antigen stimulation were not significantly above background. Differences in frequency of IL-4-producing PBMC between the case control pairs were not significant ($P > 0{\cdot}05$).

Table 2. Frequency of IFN-γ and IL-4 secreting cells (mean no. 10^{-5} peripheral blood mononuclear cells (PBMC) and 95% CI) by ELISPOT following stimulation of PBMC for 48 h *in vitro*

Antigen	Cytokine	Patients	Controls
PHA	IFN-γ	38·5 (28·6–51·9)	39·1 (27·7–55·4)
	IL-4	17·4 (10·8–28)	7·3 (4–13·3)
EB	IFN-γ	14·9 (11·7–19·2)	16·4 (12·7–21·1)
	IL-4	7 (4–12·3)	3·9 (2·5–6·2)
MOMP	IFN-γ	9·9 (7·2–13·6)	11·8 (8·7–19·1)
	IL-4	5·3 (2·9–9·5)	5·5 (3·4–8·8)
hsp60	IFN-γ	10·9 (7·7–15·3)	12·9 (8·7–19·1)
	IL-4	9·1 (5·3–15·4)	5·4 (3·4–8·8)
Unstim.	IFN-γ	9·5 (7·4–12·3)	9·8 (7·9–12·3)
	IL-4	5·7 (3·3–9·8)	4·4 (2·7–7·1)

PHA, Phytohaemagglutinin; EB, serotype A elementary bodies; MOMP, recombinant serotype A major outer membrane protein; hsp60, serotype A heat shock protein 60; unstim., unstimulated cells (medium alone + PBMC). Responses to purified protein derivative were not analysed by ELISPOT.

RT-PCR: cytokine gene transcription in PBMC

A β-actin PCR product of the appropriate size was detected in all but three of 372 samples isolated; these three were excluded from further analysis. Table 3 shows the proportion of subjects in each group in whom cytokine-specific mRNA was detected in response to mitogen and antigens. PHA induced mRNA for all cytokines most frequently, although not in all subjects. Messenger RNA specific for both IFN-γ and IL-10, but not IL-4, was identified in unstimulated cells from some subjects. IL-4-specific mRNA could only be detected in the patient group following stimulation with chlamydial antigens, but the proportion responding was not significantly above background. IFN-γ- and IL-10-specific mRNA was also generally more frequently detected in the patient group, but these differences were not statistically significant ($P > 0{\cdot}05$).

Table 3. Results of reverse transcriptase-polymerase chain reaction (RT-PCR) for cytokine mRNA isolated from 2×10^6 peripheral blood mononuclear cells (PBMC) of subjects with severe scarring trachoma and endemic controls 48 h after simulation with *Chlamydia trachomatis* antigens, mitogen or purified protein derivative (PPD)

Antigen	Cytokine	Patients no. positive /no. tested	Controls no. positive /no. tested	Odds ratio	95% CI	*P*
PHA	IFN-γ	22/29	23/29	0·86	†	>0·05
	IL-4	21/29	18/29	1·6	0·3–7·25	>0·05
	IL-10	24/30	18/30	2·2	0·64–5·92	>0·05
PPD	IFN-γ	17/30	11/30	2·5	0·63–9·9	>0·05
	IL-4	4/28	1/28	4	0·19–20·8	>0·05
	IL-10	9/30	9/30	1	†	>0·05
EB	IFN-γ	19/30	16/30	1·33	0·37–4·8	>0·05
	IL-4	3/26	0/26	NC	NC	NC
	IL-10	22/30	17/30	1·67	0·44–6·48	>0·05
MOMP	IFN-γ	16/30	7/30	3·25	0·99–6·54	>0·05
	IL-4	2/26	0/26	NC	NC	NC
	IL-10	20/30	15/30	1·63	0·02–122	>0·05
hsp60	IFN-γ	11/30	6/30	2·25	0·54–9·43	>0·05
	IL-4	3/28	0/28	NC	NC	NC
	IL-10	16/30	9/30	2·75	0·76–9·89	>0·05
Unstim	IFN-γ	6/28	3/28	1·06	0·02–16·35	>0·05*
	IL-4	0/28	0/28	NC	NC	NC
	IL-10	7/28	2/28	2·43	0·14–38·57	>0·05*

Results are expressed as the number of samples in which a PCR product of the correct size could be visualized after ethidium bromide staining of agarose gels. NC, Could not be calculated; 95% CI, 95% confidence interal. *P-value calculated by Fisher's exact test. †95% CI for OR could not be calculated.

PHA, Phytohaemagglutinin; EB, serotype A elementary bodies; MOMP, recombinant serotype A major outer membrane protein; hsp60, serotype A heat shock protein 60; unstim., unstimulated cells (medium alone + PBMC).

Association of CMI response to C. trachomatis *and HLA-DRB1, -DQB1 type*

Thirty-eight subjects from the case control pairs were HLA-DRB1- and -DQB1-typed by PCR-SSO. Twenty-three subjects had scarring disease and 15 were controls. Associations were sought between CMI response and presence/absence of any HLA-DRB1, or -DQB1 type which was found in six or more of the subjects, i.e. DRB1*04 (DR4) $n = 6$, *11 (DR11) $n = 10$, *1304 (DR13) $n = 13$, DQB1*02 (DQ2) $n = 12$, *0301 (DQ7) $n = 24$, *0302 (DQ8) $n = 6$, *0501 (DQ5) $n = 9$, and *06 (DQ6) $n = 13$.

Significant associations were found with two HLA-DRB1 alleles and a single HLA-DQB1 allele and are listed in Table 4. No significant associations were found for HLA-DRB1*04, HLA-DQB1*02, *0301, *0302, and *0501 and any of the immune responses measured ($P > 0{\cdot}05$).

DISCUSSION

We have previously reported a relative depression in lymphocyte proliferative responses to antigens of *C. trachomatis* in subjects with persistent clinical signs of inflammatory trachoma and in subjects with severe trachomatous scarring [9,10]. Since we found high anti-chlamydial antibody levels in the latter group, we suggested that failure to clear mucosal chlamydial infection, and the development of scarring sequelae, were associated with relative over-stimulation of the Th2 cellular immune response by chlamydial antigens. We tested this hypothesis by performing assays for Th1 and Th2 cellular immune responses to chlamydial antigens in subjects with scarring trachoma and in age-, sex- and location-matched controls.

The following findings were consistent with our hypothesis: incubation with hsp60 led to increased secretion of IFN-γ (a Th0/Th1 cytokine) by PBMC of control subjects but not by PBMC of those with conjunctival scarring; incubation with MOMP increased the number of IFN-γ-producing cells only in the control group; incubation with hsp60 increased the number of IL-4-producing cells (a marker of Th2 responses) among patients with scarring but not in the control group; after incubation with chlamydial antigens, IL-4-specific mRNA was only detected in subjects with conjunctival scarring.

We were unable to detect significant differences in other parameters of Th1/Th2 responses between the patient and control groups. In part, this may have been due to the small sample size imposed by logistic, financial and ethical considerations, and the consequent high chance of a type II error. Preliminary experiments established that 48 h post-stimulation was the optimum time point to select cells for ELISPOT and mRNA phenotyping (data not shown). Previous studies established that in these cultures $CD4^+$ T cells were the main subset which were responding [13,20]. However, a second constraint on this study was that only a limited number of PBMC were available from each subject, so that it was only possible to measure frequencies of cytokine-producing cells and extract RNA at a single time point and antigen dose. A further constraint was the non-quantitative nature of the RT-PCR assay,

Table 4. Summary of significant associations found between HLA class II alleles and cellular immune responses

HLA allele	Type of response	Antigen	Values (mean and 95% CI) *a*	Values (mean and 95% CI) *b*	*P*
DRB1*11	Increased IFN-γ secretion (pg/ml)	EB	491 (260–927)	193 (107–351)	0·047
DRBI*1304	Increased proliferation (SI)	hsp60	2·95 (2·15–4·05)	1·81 (1·45–2·26)	0·02
DQB1*06	Reduced proliferation (SI)	EB	10·5 (6·8–16·1)	20·2 (14–29·1)	0·03
DQB1*06	Increased frequency of IL-4-producing cells (mean no. 10^{-5} PBMC)	hsp60	10·3 (5·1–20·8)	4·4 (2·8–7·1)	0·05

a, Mean values obtained in samples from subjects which were positive for that particular HLA allele; *b*, mean values obtained from subjects in which that particular HLA allele was absent; SI, stimulation index; EB, *Chlamydia trachomatis* elementary bodies; hsp60, *C. trachomatis* heat shock protein 60.

which may have obscured differences between groups. Nevertheless, non-quantitative RT-PCR for cytokine message can be informative, as many other groups have shown [11,21]. In addition, we have not assigned Th1/Th2/Th0 phenotypes but assessed the ability of chlamydial antigens to induce PBMC, from trachoma patients and controls, to produce different cytokines which are representative of these cell types.

The low levels of IL-4-secreting cells and mRNA induction indicate that there are few *Chlamydia*-specific IL-4-producing cells in the PBMC population. These cells may have been sequestered or may be short lived in the peripheral circulation. Alternatively, under the culture conditions used in this study, IL-4-secreting cells may be down-regulated by other factors produced in the mixed cell culture. Indeed it was not possible to detect IL-4 in the cell-free supernatant of PBMC cultures. However, consistent trends in the frequency of IL-4-producing cells were evident in response to mitogen, EB and hsp60. Mean levels of IL-4-secreting PBMC were greater in response to these stimuli in the group with scarring trachoma compared with controls. Taken together with the finding that mRNA for IL-4 was only found in samples from subjects with scarring disease, it may suggest that IL-4 plays an important role in the promotion of the scarring fibrotic changes in the conjunctiva. The role of Th-2-like cells and cytokines, in particular IL-4 and IL-12, in the development of fibrosis following infection is being increasingly realised [22,23]. Perhaps the pathological changes due to chronic ocular infection with *C. trachomatis* arise by a similar mechanism.

Large individual variations were evident in the response to chlamydial antigens. Since immune responses are under the control of HLA genes, these variations may be due to differences in HLA class II genotype among subjects. We examined the influence of genotype on the degree or extent of cellular response. In order to reduce the chance of type I error the statistical analysis was restricted to alleles which were common in this sample. In addition, we sought associations only with chlamydial antigens. Perhaps most interesting is the association of HLA-DQB1*06 with decreased proliferation to EB and an increased frequency of IL-4-producing cells to hsp60. This may suggest that stimulation with hsp60 selectively activates IL-4-secreting cells in subjects who are HLA-DRB1*1304-positive. This is not without precedent, since Pfeiffer *et al.* [24] found that MHC type controlled Th1/Th2 activation in response to a peptide from type IV collagen. However, although we observed associations, it should be noted that these were not highly significant given the number of comparisons and tests performed (i.e. 8 HLA types $\times$ 4 assay types $\times$ 3 chlamydial antigens tested). These associations should therefore be treated with caution.

There were no significant associations between HLA types and clinical signs, and the small number of individuals typed does not allow meaningful comparison with allelic frequencies found in the Gambian population. However, these associations may warrant further study and require independent validation with a larger sample size. Other authors have reported HLA class II restriction of T cell clones to chlamydial EB, but these were generated from only a few sensitized laboratory donors [25,26]. Our results represent the first study of HLA class II restriction and several measures of cellular immunity to both whole and recombinant chlamydial antigens in a community in which exposure to *C. trachomatis* is endemic. In the mouse, antibody responses to both hsp60 and hsp70 have been shown to be H-2-linked [27]. Furthermore, the mortality rate following mouse pneumonitis agent infection has been shown to be strain-dependent [28] and this correlated with reciprocal Th1/Th2 immune responses in different strains. These observations may further support our findings. In our study population, however, we found no evidence of strong associations between HLA type and immune response to chlamydial antigens.

Our results are consistent with the hypothesis of reciprocal Th1/Th2 activity. The relative increase in Th2 responses could lead to down-regulation of protective Th0/Th1/$CD8^+$ cells and hence an inability to clear ocular chlamydial infection. This may contribute to the induction of persistent or latent forms of *C. trachomatis* which continue to stimulate pathological changes and promote fibrosis [5,22,23,29,30]. Future studies should investigate the possible link between Th1/Th2 imbalance, down-regulation of protective host responses, and its contribution to the pathogenesis of trachomatous scarring.

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