Characterization of B-Cell Responses to *Chlamydia trachomatis* Antigens in Humans with Trachoma

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Trachoma is a chronic keratoconjunctivitis caused by intra-cellular infection of epithelial cells with *Chlamydia trachomatis* and is the leading cause of infectious blindness, with 12 million new cases of trachomatous blindness predicted within 30 years (40). Trachoma is usually associated with the ocular serovars A, B, Ba, and C. Active trachoma, which is usually associated with evidence of ocular *C. trachomatis* infection, may range from a mild asymptomatic inflammation with collections of immune cells visible on the upper tarsal conjunctiva (follicular trachoma [TF]) to an intense inflammatory response in which most of the tarsal plate is obscured by capillary congestion (intense trachoma [TI]). Repeated ocular infections cause scarring of the conjunctiva (scarring trachoma [TS]), inversion of eyelids and eyelashes (trichiasis), and ultimately blindness follows corneal abrasion by intruded lashes.

The interactions between cellular and humoral immune mechanisms involved in clearance of human chlamydial infection and in development of protection or adverse sequelae are poorly understood. That some degree of protective immunity occurs is suggested by observations that active trachoma is a disease of children (3, 44). *C. trachomatis*-specific antibody responses in serum and local secretions are found following natural infection (7). Serum antichlamydial immunoglobulin G (IgG) antibodies are long lasting (8, 10) but do not confer protection against ocular challenge of naive guinea pigs in passive transfer experiments (49). However, it has been suggested that local secretory IgA (SIgA) responses are associated with protection from ocular infection (5). SIgA responses were inversely correlated with shedding of infection in chlamydial genital infection (7) and may thus play a role in neutralizing infectious *C. trachomatis* elementary bodies (EBs) both in the infectious challenge and released from epithelial cells during ongoing infection. It is likely that cell-mediated immunity (CMI) plays the key role in eradication of established intracellular infection, as *C. trachomatis* is an obligate intracellular organism. Animal studies have confirmed the central role of CD4+ T cells (27) and NK cells in controlling chlamydial genital infection (50), and proinflammatory cytokines associated with cell-mediated responses are increased in trachoma (4, 6). Gamma interferon is chlamydiostatic in vitro (28), and studies using gene-knockout mice have clearly demonstrated the requirement of interferon gamma rather than SIgA for eradication of established intracellular genital infection (24). While the incidence and duration of active trachoma episodes decrease with age, episodes are more likely to be associated with intense inflammation (1). Attempts to prevent trachoma by vaccination with whole organisms in the 1960s were unsuccessful, as while some subjects were protected, there was a suggestion that other vaccinated subjects developed enhanced disease (42). In a monkey model, immunization induced only more severe disease was seen in some animals on heterologous challenge (47). This adverse hypersensitivity is not serotype specific and persists longer than protective immunity (46). It has been suggested that hypersensitivity to chlamydial heat shock protein 60 (hsp60) might underlie these observations, while other antigens such as the major outer membrane protein (MOMP) may induce protection (32, 48).
It is desirable to use techniques that can evaluate the immune response during a specific episode of infection without bias from previous episodes, as each episode will induce a balance of cellular and humoral immunity which depends on the history of previous infections, the dose and nature of infecting organism, and host factors which may change with time. Interpretation of serological responses is hampered by the relatively long persistence of serum antibody, and titers are a cumulative synthesis of previous responses. Proliferative T-cell assays specifically identify memory T cells which may have been generated in the distant past. SLgA has the benefit of a short duration of response, but antigen-specific responses are difficult to quantitate in most mucosal secretions, and in the tear film in particular, due to problems with standardizing collection of low-volume samples, differences in flow rates, the short-lived nature of SLgA responses (15), and degradation by bacterial proteases. The enzyme-linked immunospot (ELISPOT) assay detects short-lived immature plasmablasts induced by antigen presentation within the preceding days or weeks (18, 19, 25, 30). When secretions from accessible sterile mucosal sites with relatively constant high-volume secretion rates (such as small bowel) have been studied, the enumeration of circulating antigen-specific plasmablasts by the ELISPOT assay correlates well with antigen-specific SLgA levels (16, 21). We describe here for the first time application of the ELISPOT assay to quantify antigen- and isotype-specific B-cell immune responses in peripheral blood and urine associated with different clinical features of trachoma in subjects from two trachoma-endemic villages in The Gambia.

MATERIALS AND METHODS

Subjects. The study protocol was approved by the Ethical Committee of the MRC Laboratories, Fajara, The Gambia. Trachoma study subjects consisted of children and adults resident in the Gambian villages of Jali and Berendeng, an area endemic for trachoma as described previously (2, 3). Subjects were examined and graded by a trained ophthalmic assistant and checked by an experienced observer. Trachoma was graded clinically according to World Health Organization criteria. Sixty children were divided into three groups: 19 with no signs of active ocular disease (NS group), 36 with trachomatous inflammation of follicular grade (TF group), and 5 with trachomatous inflammation of intense grade (TI group). Ages ranged from 3 to 14 years for the NS group (10 years for the TF and TI groups). Seventeen age- and sex-matched pairs of adults with no signs of active trachoma (NS group) or the presence of scars alone (NS group), 36 with trachomatous inflammation of follicular grade (TF group), and 5 with trachomatous inflammation of intense grade (TI group). Ages ranged from 18 to 60 years for the NS group (30 years for the TF and TI groups). Six months or less of follow-up were included for the children who were enrolled in the NS group.

ELISPOT assay. ELISPOT assay (17, 41) as described previously (26) and adapted from the method of Pal et al. (34) was used to detect antigen-specific antibody-secreting cells (ASCs) in peripheral blood and urine. Peripheral blood mononuclear cells (PBMCs) were obtained by ficoll-hypaque (Sigma, Poole, England) discontinuous gradient centrifugation of 5 to 10 ml of heparinized whole blood obtained by antecubital fossa venipuncture. Urine mononuclear cells (UMNCs) were separated from 40 to 60 ml of urine (obtained on the same occasion as blood) voided into sterile containers and centrifuged at 800 g for 15 min at 4°C. PBMCs or 10^6 UMNCs assayed. Recombinant MOMP of C. trachomatis serovar A (and F in some cases) and recombinant C. trachomatis serovar L2 lps60 were purified as previously described (36).

ASC responses to chlamydial antigens. (i) U.K. subjects. In the development of the assay, we were unable to detect ASCs secreting antibody to any of the chlamydial antigens used in 13 asymptomatic healthy U.K. subjects (mean age, 34.0 years, range, 25 to 49 years), 9 patients with active Salmonella infection, or 6 patients with previous exposure to C. pneumoniae (evidenced by IgG titer greater than 1:64 by microimmunofluorescence, but without evidence of acute respiratory disease). In contrast, ASCs were detected in all 35 subjects attending the...
responses in blood per 2
numbers with detectable IgG or IgM responses were generally
able IgA ASC response to MOMP followed a pattern similar to
compared to the NS group).

The numbers of IgA ASCs to recombinant serovar F and A
were simultaneously enumerated in 55 children, and
there was no significant difference in IgA ASC numbers in any
of the three study groups (data not shown). The IgA subclass
distribution of trafficking anti-MOMP ASC response in the NS
group was 58% IgA1 and 42% IgA2. In the TF group, the ratio
was 72% IgA1 to 28% IgA2. Responses in the TI group were
too low to determine subclass ratio.

(iii) ASC responses of children to hsp60. The ASC response
to chlamydial hsp60 (Fig. 1, center panel) was similar to that
for MOMP in terms of absolute numbers of ASCs, isotype
dominance, and pattern of response between groups. There
was a slightly higher IgA ASC response in TF compared to NS,
and this was the dominant isotype in TF. The anti-hsp60 ASC
response for all isotypes was almost completely absent in TI,
and this was statistically significant for IgA (P = 0.04 compared
to TF; P = 0.05 compared to NS).

(iv) ASC responses of children to whole EBs. The pattern of
ASC response to whole EBs (Fig. 1, lower panel) was generally
similar to that seen with recombinant proteins, with IgA the
dominant isotype in TF (P = 0.05). The distribution of trafficking anti-MOMP ASC response in the NS
group was 72% IgA1 to 28% IgA2. Responses in the TI group were
too low to determine subclass ratio.

(v) ASC responses of adults to MOMP. Anti-MOMP IgA
ASCs were detectable in both scarred (TS) and unscarred (NS)
groups (Fig. 2, top). IgG and IgM responses were much lower,
especially in the nonscarred subjects (P = 0.006 and 0.005 for magnitude of response).

(vi) ASC responses of adults to hsp60. Anti-hsp60 IgA ASCs
were detectable in both scarred (TS) and unscarred (NS)
groups (Fig. 2, center). As with MOMP, the magnitudes of the
IgG and IgM responses were much lower, especially in the
nonscarred subjects (P = 0.01 and 0.02 for magnitude of response).

(vii) ASC responses of adults to EBs. Anti-EB IgA ASCs
were detectable in both scarred (TS) and unscarred (NS)
groups (Fig. 2, bottom). As with MOMP and hsp60, IgG and
IgM responses were much lower, especially in the
nonscarred subjects (P = 0.004 and 0.003 for magnitude of response).

(viii) Serum IgA and IgG responses to MOMP. The serum
IgA and IgG responses to MOMP determined by ELISA are
shown in Fig. 3. In contrast to the ASC responses, the anti-
MOMP IgA activity in the children was high in all groups,
including those with TI. Interestingly, both adult groups had
the highest anti-MOMP IgA activity, in keeping with the pro-
gressive acquisition of immunity with advancing age. Although
anti-MOMP IgM activity was lowest in the children with TF
and highest in adults with TS, there were no significant differ-
ces between clinical groups.

Detection of chlamydial infection by PCR. The PCR assay
readily detected C. trachomatis DNA in U.K. subjects with
positive urethral swabs, reconfirming the sensitivity of the as-
say (2). However, no chlamydial DNA could be detected in the
urine of any Gambian children in the study, making chlamydial
genital infection an unlikely cause of the urine ASC response
observed.
DISCUSSION

In this study, we have demonstrated for the first time trafficking C. trachomatis-specific ASCs during human chlamydial ocular infection. The key feature of the ELISPOT assay is that it detects only cells already spontaneously secreting antibodies at the time of sampling. The short assay duration means that resting B cells (naive or memory cells from previous responses) are not activated. ASCs detected by the ELISPOT assay are essentially immature plasmablasts circulating in blood during tightly regulated trafficking between sites of immune induction and final tissue location. They characterize the activated B-cell response (from naive or recirculating memory B cells) to antigen presentation within preceding days or weeks. In nonmalignant situations, spontaneous ASCs are present in blood for only a week or so before homing to tissue, where they survive for about 60 days (18–20, 30). They are replaced in blood by long-lived, recirculating resting memory B cells (43), which can be detected only by prolonged stimulation with mitogens (3 to 5 days) or antigen (9 to 10 days) prior to the ELISPOT assay. Gut-based studies show that IgA ASCs detected after mucosal immunization express a mucosa-specific phenotype (37), and numbers correlate well with antigen-specific SIgA responses in secretions (16, 21). The detection of trafficking antigen-specific ASCs provides an excellent surrogate for cellular immune events at inaccessible sites. Furthermore, by standardizing results to the number of cells assayed, the ELISPOT assay permits antigen-, class-, and subclass-specific quantification of response, unlike ELISA but analogous to limiting dilution assay of proliferative T-cell responses (12, 13). While trafficking chlamydia-specific B cells have been demonstrated in a monkey model of ocular chlamydial infection, to date no studies have reported B-cell responses in human infection.

Serological studies have identified MOMP as the dominant chlamydial antigen in humoral immunity (11). When used in the ELISPOT assay, whole chlamydial EBs present a range of antigens in which MOMP probably predominates. It is possible that recombinant MOMP used in our assay is not in the native conformation expressed on EBs. This, together with the only partial cross-reaction of L2 serovar EBs used as coating antigens with serovar A and B organisms causing infection, probably explains the apparent discrepancy between absolute number of ASCs to EBs versus recombinant MOMP. However, we were reassured by the reproducibility of results when recombinant antigens from different sources were directly compared. The role of other antigens such as lipopolysaccharide or OMP2 remains to be elucidated, but the assay could easily be adapted to study these antigens, as well as peptides containing neutralizing and serovar-specific epitopes.

Ocular C. trachomatis infection is endemic in these Gambian villages, and children are repeatedly exposed. For ethical reasons, we were able to study children at only one time point, and therefore the ASC responses observed represent a snapshot of the evolving immune response to discrete episodes of infection. Between 53 and 57% of children with no signs of active ocular infection had IgA ASCs to the chlamydial antigens used, which indicates the high rate of exposure to infection in
this age group. These children may have been developing active disease, recovering from recent infection, or infected in other sites such as the nasopharynx. The development of active follicular disease was associated with an increased percentage of children having detectable IgA ASC responses to MOMP and a higher mean number of MOMP-specific IgA ASCs. The percentage of children with a detectable IgG ASC response fell for all antigens when follicular disease developed, and the mean number of IgG and IgM ASCs to the three antigens studied either fell or remained unchanged. The specific boosting of IgA ASCs during active follicular disease with a relative fall in IgG is highly suggestive of a mucosal, TH2-type response. The ability of the mucosal immune system to maintain SlgA responses on the background of cell-mediated responses and a TH1 pattern of cytokine secretion such as in TF (6) has been found in mice and is probably due to the simultaneous production of interleukin-10 and interleukin-6 (45). Whether these mechanisms are active in human trachoma remains to be determined.

A striking observation in this study was the dramatic reduction in ASC response associated with intense ocular disease in the children. The number of chlamydia-specific ASCs of all isotypes and to all antigens was reduced, but most significant was the fall in IgA ASCs. The similar anti-MOMP serum IgA response in children in all three clinical groups suggests that the low IgA ASC response associated with intense ocular inflammation does not represent an inherent deficiency of IgA. It might indicate a polarization towards a proinflammatory, TH1-type response, as suggested by cytokine studies (6), which temporarily suppresses IgA-inducing mechanisms. Alternatively, IgA ASCs may become sequestered in the conjunctiva. As we studied only one time point, it will be important to carry out longitudinal studies to determine whether some children with recurrent disease repeatedly suppress the IgA ASC response. Such suppression might allow the clearance of infection by CMI but conversely enhance tissue damage by the absence of SlgA which can bind EBs without activating complement. The concomitant suppression of ASC response to hsp60 does not suggest a specific role for hsp60 in the immune responses to intense disease. The relatively high number of anti-MOMP IgA2 ASCs in children with no ocular inflammation is compatible with a mucosal origin (31), and the increased IgA1 ASCs in TF is compatible with the distribution of IgA subclasses in lachrymal gland secretions (31) and selective induction of IgA1 by peptide antigens. Studies using different antigens and possibly epitope-specific peptides are required to further characterize these observations.

Circulating ASCs could be detected in adults both with and without evidence of scarring. None of the adults had evidence of active ocular disease, and ASCs may have been induced by ocular or genital infection. The magnitude of IgA ASC responses to all three antigens was higher in the scarred group, but the difference was not significant. SlgA responses may therefore become established even in older children and adults who develop scarring, and serum anti-MOMP IgA levels were higher in the adults, suggesting progressively acquired immunity. There was a trend for more adults without scarring (81%) than those with scarring (61%) to have an IgA1 ASC response to MOMP. In contrast, there was an opposing trend for more adults with (70%) than without (53%) scarring to have anti-hsp60 IgA ASC responses. This reversed ratio of the IgA ASC response to MOMP and hsp60 and the increased magnitude of the IgG ASC response to hsp60 in scarred adults is intriguing, suggesting an increased priming of the immune system to hsp60 in the scarred group. As with serology, ASC responses to hsp60 may represent effects of tissue damage and may not in themselves causally contribute to scarring. Although the differences did not reach significance, these data are nevertheless compatible with serological studies which have correlated the presence of serum anti-hsp60 IgG responses with pathological sequelae such as scarring trachoma (35) and ectopic pregnancy (9). SlgA does not bind complement and is therefore noninflammatory, but by blocking attachment of chlamydial EBs could act with CMI, either during initial infection or following release of intracellular organisms from lysed epithelial cells. Although specific SlgA responses are relatively short lived, the presence of memory B and T cells in the mucosal site will enable a rapid anamnestic response to rechallenge. Thus, a balanced immune response with vigorous CMI to control intracellular infection or lyse infected cells, and high levels of SlgA to neutralize released EBs and localize infection, might result in efficient eradication of infection with limited inflammation. Blindness from trachoma occurs when repeated intense inflammatory episodes of chlamydial disease lead to scarring, inversion of the eyelid, and abrasion of the cornea by the eyelashes. If an individual develops a tendency to respond to ocular infection with a vigorous proinflammatory response, without neutralizing SlgA, then repeated episodes may lead to scarring. Once corneal abrasion develops, recurrent nonchlamydial infections may also induce proinflammatory responses and aggravate tissue damage. It is interesting that while episodes of trachoma become less frequent and of shorter duration in children as they grow older, each episode is more likely to be intense (1). It may be that children who develop CMI without appreciable SlgA gain immune protection, but at the cost of enhanced tissue damage with each episode of infection. Trachoma therefore provides an excellent model to characterize immune events leading to protection or pathology, as the inflammatory response in the eye can so readily be observed and clinically graded.

In a monkey model of conjunctival infection, more C. trachomatis-specific IgA ASCs were detected by ELISPOT assay in conjunctival biopsies than in peripheral blood (34), but nonocular mucosal sites were not studied. Our observation that anti-MOMP ASCs were readily detectable in urine of children without evidence of genital chlamydial infection detectable by PCR is the first demonstration of the integration of ocular and genital mucosa in a common mucosal immune system in humans. These B cells may have entered the urine passively across the kidney glomeruli; however, the relative number of antigen-specific IgA ASCs (standardized in the assay relative to total mononuclear cells studied) in the urine was 8 to 25 times higher than in blood taken at the same time, which suggests specific homing. That relatively fewer subjects had detectable urine than blood ASCs may indicate the lower sensitivity of urine (which probably relies on cells being washed off the mucosal epithelium) or may indicate the kinetics of the response whereby cells appear first in blood and then home to mucosal sites. Longitudinal studies of urine and blood responses will be required to address this matter. The potential integration of ocular and genital mucosal surfaces is probably mediated by shared cell surface integrins such as α6β1 integrin and addressins such as MAdCAM-1 associated with gut homing of lymphocytes (39). Shared addressins on cells mediating inflammatory responses may explain phenomena such as reactive arthritis and conjunctivitis following genital chlamydial infection (38).

In conclusion, we have demonstrated for the first time the presence of C. trachomatis-specific B cells in the circulation of children at different stages of trachoma. Mild, follicular trachoma is associated with an increased IgA cellular response to MOMP, hsp60, and whole EBs, suggesting a mucosal-type
response. In contrast, intense trachomatous inflammation was associated not with a further increase in the B-cell response but with a reduction of peripheral blood B-cell responses to all antigens and of all three isotypes. In contrast, serum anti-MOMP IgA levels were similar in the childhood groups, suggesting suppression of IgA cellular responses during intense disease. Thus, protective vaccines against *C. trachomatis* should induce a good cell-mediated and IgA response to a broad range of chlamydial antigens. Vaccines may have to be delivered via a mucosal route (14, 23, 33) to maximize neutralizing IgA responses and prevent excessive polarization of immune recall to proinflammatory responses. Our observation that the ocular and genital mucosal tissue may be integrated into a common mucosal immune system in humans offers support for this strategy.

We are characterizing phenotypic markers such as L-selectin and α4β7 integrin on B cells detected in urine and blood of U.K. subjects with active genital chlamydial disease, and extension of these studies to ocular infection will help to determine the contribution of the eye and genital tract to a common mucosal immune system. Such data will have major implications for vaccine delivery to mucosal sites to induce immune protection in the eye. Further longitudinal studies to characterize patterns of response in the transition from frequent mild trachoma to infrequent intense disease and scarring will be required to confirm the association of a reduced neutralizing IgA responses with the processes leading to scarring and blindness.

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