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

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Received 2 May 1997/Returned for modification 15 July 1997/Accepted 19 September 1997

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 intertuned 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 chlamydiosiatic 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 short-lived serotype-specific immunity to ocular challenge, and 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 quantify 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 immunosorbent assay (ELISPOT) assay detects short-lived immature plasma blasts 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 plasma blasts 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 urea 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 Berending, 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) (mean age, 8.9 years; ranges, 8 to 11 years for the NS group and 8 to 10 years for the TF and TI groups). Seventeen age- and sex-matched pairs of adults with no signs of active trachoma (NS groups) or the presence of scars alone (NS groups) were studied. None of the adults with TS had signs of active disease, but six had trichiasis.

Four groups of volunteers attending St. George’s Hospital were used in the development of the assay: healthy asymptomatic adults with no current or past history of ocular or genital chlamydial infection; patients with active enteric Salmonella infection; patients with previous exposure to C. pneumoniae evidenced by high serum microimmunofluorescence antibody titers to C. pneumoniae; and patients attending Gento-Urinary Medicine (JUM) clinic with active genital chlamydial infection confirmed by C. trachomatis-specific PCR (2) or direct immunofluorescence (Microtrack; Syva UK, Maidenhead, England) of urethral or cervical swabs.

Chlamydial antigens. Elementary bodies of C. trachomatis serovar L2 concentrated by continuous gradient centrifugation were obtained from OCM Concepts, Toms River, N.J. and purity was confirmed by electron microscopy. Recombinant MOMP of C. trachomatis serovar A (and F in some cases) and recombinant C. trachomatis serovar L2 Lsp60 were purified as previously described (36).

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 Ficol-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, diluted 1:2 at 4°C and 105 UMNCs assayed. Cells were resuspended and washed twice in phosphate-buffered saline (PBS). Polystyrene 25-well plates (5 × 5 plates; catalog no. 103; Sterilin, Teddington, England) were coated with chlamydial antigens (EBs at 25 μg/ml, MOMP at 2 μg/ml and Lsp60 at 10 μg/ml) in carbonate buffer (pH 9.6) for 1 h at 4°C, then blocked with 1% (wt/vol) Hammarsten casein (BDH, Poole, England) in PBS (pH 7.4). After washing, cells were counted in a hemocytometer chamber and resuspended in RPMI 1640 with no added serum or antibiotics, and 106 cells per well were incubated in at 37°C with 5% CO2 for 18 h. Cells were removed by vigorous washing with PBS-0.05% Tween 20, and plates were incubated for 2 h at 37°C with goat anti-human γ chain or goat anti-human α chain diluted 1:250 or with goat anti-human μ chain diluted 1:250. After washing in PBS-Tween 20, wells received anti-goat IgG–alkaline phosphatase conjugate diluted 1:250. All antibodies were from Sigma Chemical and diluted in 1% (wt/vol) casein-0.05% Tween-PBS (pH 7.4). IgA subclass-specific ASCs were detected by using mouse anti-human IgA1 or IgA2 monoclonal antibodies (SeraLab, Crawley, Down, England) diluted 1:100, followed by rabbit anti-mouse IgG–alkaline phosphatase conjugate (Sigma) diluted 1:250. Bound antibody was detected by using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) substrate (Sigma). Spots representing one ASC were enumerated after 24 h at room temperature by low-power magnification. On polyester-poly carbonate plates, each spot with the size and appearance characteristic of an ELISPOT is counted as significant (22), with control wells without antigen used to confirm specificity. Results are expressed as number of ASCs per 2 × 106 PBMCs or 105 UMNCs assayed.

ELISA. The serum IgA and IgG anti-MOMP antibody response was determined by direct enzyme-linked immunosorbent assay (ELISA). Costar high-binding 96-well enzyme immunoassay plates were coated overnight at 4°C with 100 μl of MOMP (1 μg/ml) in carbonate buffer (pH 9.6) and then blocked with 200 μl of 1% casein in carbonate buffer (pH 9.6). In subsequent wells, stages received 100 μl with reagents diluted in 1% (wt/vol) casein-0.05% Tween 20-PBS (pH 7.4). All sera were assayed in duplicate in 4 to 8 doubling dilutions from 1:4, or 1:20 if high activity was detected. Each plate assayed included replicate dilutions of a reference serum with known activity. After 150 min at 37°C, plates were washed and goat anti-human γ chain-alkaline phosphatase conjugate or goat anti-human α chain-alkaline phosphatase conjugate or goat anti-human μ chain-alkaline phosphatase conjugate (Sigma) diluted 1:300 was added. After 150 min at 37°C, plates were washed and 1 mg of p-nitrophenyl phosphate (Sigma) per ml in 1 M diethanolamine-0.5 mM MgCl2–6H2O buffer (pH 9.8) was added. Plates were read at 405 nm when the reference samples reached an optical density of 1.0. The antichlamydial activity of test sera, expressed in nominal ELISA units, was determined relative to the reference serum on the same plate by parallel line regression analysis (20) as described previously (26). Briefly, in this method the mean absorbance value from the duplicate dilutions is calculated for the reference and test samples, and the background activity is subtracted. This value is plotted against the logarithm of the reciprocal of dilution, and a least-squares regression line is calculated. In this method the mean absorbance value from the duplicate dilutions is calculated for the reference and test samples, and the background activity is subtracted. This value is plotted against the logarithm of the reciprocal of dilution, and a least-squares regression line is calculated. The x-intercept of these is used to calculate the potency of the test serum relative to the reference. The process was automatically calculated by using a computer linked directly to the plate reader. The reference serum was obtained from one of the study subjects, and the same serum was used throughout. A nominal activity of 1.0 IgG ELISA units and 48 IgA ELISA units was assigned to this serum after replicate analyses showed a mean x-axis intercept of 1.017 with the IgG assay and 48 with the IgA assay.

PCR assay for detection of C. trachomatis in urine. To identify genital chlamydial infection, samples of urine were collected from children into sterile plastic containers, and 1-ml aliquots were transferred into Eppendorf tubes and stored frozen at −70°C. Samples were thawed in batches and centrifuged at 9,500 × g for 30 min, and extracts were heated at 80°C for 1 min. The urine pellets by PCR assay using previously described primers (2). The technique has been extensively used to detect ocular C. trachomatis in these Gambian villages and detects l to 10 EBs (2). As positive controls, urine samples from U.K. adults attending the GUM clinic at St. George’s Hospital with confirmed C. trachomatis genital infection were also assayed.

Statistical methods. Student’s t test (paired for age- and sex-matched adults; unpaired for children) was used to compare mean number of antigen- and isotype-specific ASCs between clinical groups. The chi-square test was used to compare numbers of subjects demonstrating an ASC response between groups, or Fisher’s exact test where expected numbers were less than 5. Two-tailed P values less than 0.05 were taken as significant.

RESULTS

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
firmed evidence of
GUM clinic with PCR- or direct immunofluorescence-con-
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 chlamydial-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 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 IgA 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.

ACKNOWLEDGMENTS

This work was supported by award to S.G.-M. of the 1996 John William Clark Award for research into the causes of blindness by the British Medical Association.

We acknowledge the helpful comments of D. Taylor-Robinson in the development of the ELISPOT assay and Lynn Hayes for provision of recombinant antigens.

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Editor: J. R. McGhee