Antigenic Specificity of Human Antibody to Chlamydia in Trachoma and Lymphogranuloma Venereum

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An understanding of the molecular basis of the humoral immune response to chlamydial infections in man requires the identification of target antigens to which antibodies are directed. The antigenic specificity of antibody from patients with lymphogranuloma venereum (LGV) or trachoma was therefore assessed by Western blotting. Surface polypeptides were first identified using purified chlamydial outer membrane complex as antigen. Antibodies in sera from patients with LGV but not from control negative sera reacted with a wide range of chlamydial surface polypeptides with molecular masses of 19, 29, 41, 58, 63 and 65 kDa. The major component of the antibody response detected by both immunoblotting and immunoprecipitation assay was directed against the major outer membrane protein (MOMP). Antibody to MOMP was species-specific on Western blotting, whereas antibody to several other polypeptides recognized common immunodeterminants on polypeptides of C. psittaci Cal-10 of equivalent molecular mass. Immunologically C. psittaci Cal-10 was more closely related to LGV strains of C. trachomatis than a guinea pig inclusion conjunctivitis strain of C. psittaci.

Trachoma sera collected from a village in southern Iran showed predominantly type-specific antibody on micro-immunofluorescence to serotype A or B trachoma agents. These sera showed a weak immune response to MOMP, a pronounced response to a polypeptide of 36 kDa and much less widespread reactivity with other chlamydial polypeptides. The lack of an immune response to SDS-stable immunodeterminants on MOMP might contribute to the susceptibility of trachoma patients to repeated cycles of ocular infection with chlamydiae.

INTRODUCTION

Lymphogranuloma venereum (LGV) and trachoma are clinically and immunologically distinct manifestations of human infection with Chlamydia trachomatis. In LGV, chlamydiae of serotypes L1, L2 or L3 invade and multiply within lymph nodes draining the genital tract, producing a strong, systemic antibody response reactive with multiple chlamydial serotypes. Hyperendemic trachoma, by contrast, is a localized ocular infection due to chlamydiae of serotypes A, B or C, which engender a weak type-specific antibody response in both tears and sera. Multiple reinfection is the norm but eventually gives rise to protective immunity as shown by the fact that trachoma is primarily a childhood disease (Schachter & Dawson, 1978). Antibody to the serotype-specific immunodeterminants offers limited protection in man or primate models to ocular challenge with organisms of homologous serotype (Monnickendam & Pearce, 1983). However, severe disease was observed in subjects immunized experimentally with whole trachoma organisms who subsequently became infected with organisms of heterologous serotype. The implication was that repeated ocular exposure to cross-reactive...
chlamydial antigens in a region of hyperendemic trachoma transmission gave rise to damaging immune hypersensitization leading ultimately to severe scarring and blindness (Schachter & Dawson, 1978). Empirical vaccination having failed, any further vaccine trials necessitate an understanding of the contribution of individual chlamydial surface antigens to the human immune response.

Several potentially important chlamydial surface antigens have already been identified. The chlamydial major outer membrane protein (MOMP) functions as a porin (Chang et al., 1982; Bavoil et al., 1984), comprising some 60% of the protein at the surface of the infectious elementary bodies (EB). Serotype-, subspecies- and species-reactive immunodeterminants have been identified on this protein with monoclonal antibodies (Stephens et al., 1982; Matikainen & Terho, 1983) and account for much of the serological cross-reactivity between chlamydial strains observed by micro-immunofluorescence (micro-IF) (Caldwell & Schachter, 1982). Some EB surface proteins are cysteine-rich, forming disulfide-linked oligomers, which maintain the essential structural rigidity of the EB in the absence of peptidoglycan (Hatch et al., 1984). A number of other surface proteins of unknown function have also been identified (Wenman et al., 1982a, b). In addition, an endotoxic lipopolysaccharide (LPS) gives rise to complement-fixing antibody reactive with C. psittaci, C. trachomatis and the LPS core of other Gram-negative bacteria (Volkert & Matthiesen, 1956; Nurminen et al., 1984).

The contribution of these surface antigens to the human immune response can be identified by immunoblotting or by immunoprecipitation. Newhall et al. (1982) used Western blotting in a study of sera from patients with chlamydial genital tract infection. Antibody to a protein of 62 kDa was common in patients with infection but widespread reaction of other control sera from non-chlamydial infected individuals was identified to other antigens including MOMP. This paper describes the antigenic specificity of sera from patients with trachoma and LGV investigated by Western blotting and by radioimmunoprecipitation.

METHODS

Chlamydiae. The C. trachomatis strains used were A/SA1/OT, B/TW5/OT, C/UW1/OT, E/DK20/ON and L1/440/LN. C. psittaci strains were meningopneumonieumatis Cal-10 and guinea pig inclusion conjunctivitis (GPCP) A10. C. trachomatis strains SA1, TW5 and UW1 were grown in BGMK cells (Hobson et al., 1982) in Dulbecco's minimal essential medium in the presence of 1 μg cycloheximide ml⁻¹. All other chlamydial strains were grown in cycloheximide-treated McCoy cells, prepared and purified as previously detailed (Salari & Ward, 1981). Chlamydial protein concentrations were determined by comparing the A₂₈₀ of samples dissolved in 0-1 M-NaOH with a standard curve based on a known mass of bovine serum albumin (BSA).

Sera. Monoclonal antibodies were prepared by conventional procedures using purified C. trachomatis L1/440/LN as antigen and hybridomas derived from Balb/c mouse spleens and NS1 cells. Sera from patients with the typical clinical features of LGV and high antibody to LGV strains of C. trachomatis originated from the laboratories of Dr R. Ballard in South Africa and Dr D. Mabey in The Gambia, West Africa, to whom we are most grateful. Sera from patients with typical active trachoma (T1, T2 or T3) and positive antibody to C. trachomatis serotype A, B or C on micro-IF were from the collection of sera at the Institute of Ophthalmology, London. These patients were from the village of Sar-rig in southern Iran, a region with hyperendemic blinding disease. Trachoma agents were isolated from approximately half of the patients. Psittacosis sera were selected from patients with atypical pneumonia responsive to tetracycline who demonstrated positive antibodies to a pool of C. psittaci strains by micro-IF. Rabbit antiserum was from an animal hyperimmunized with C. trachomatis L2/434/Bu in Freund's adjuvant (Difco) over several months (Ward & Murray, 1984). Samples of sera were thawed once, mixed with an equal volume of glycerol as anti-freeze, then stored liquid at −20°C.

Serology. Micro-IF was done at the Institute of Ophthalmology, London, as previously described (Treharne et al., 1977).

Polyacrylamide gel electrophoresis. Polyacrylamide slab gels, 0.75 mm thick, comprised a running gel with a linear gradient of 10 to 25% (w/v) acrylamide, a stacking gel of 5% (w/v) acrylamide and the discontinuous buffer system of Laemmli as previously described (Salari & Ward, 1981). Chlamydiae were derivatized by boiling for 5 min in dissociating buffer and electrophoresed for 16 h at 175 V and 4°C at a starting current of 25 mA. Molecular mass standards (Sigma) were myosin, β-galactosidase, phosphorylase b, catalase, actin, DNAase, trypsinogen and myoglobin.

Protein iodination. A single Iodobead (Pierce Chemical Co.) was incubated for 5 min at room temperature with a reaction mixture of 100 μg protein A (Sigma), 1 mCi (37 MBq) Na¹²⁵I (Amersham) and 100 μl PBS (Dulbecco A,
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Oxoid). Unbound iodine was removed from labelled protein by gel filtration on Sephadex G25. Specific activity was 2-4 μCi (μg protein)^{-1}. Bolton and Hunter iodination was done as recommended in the accompanying product leaflet from Amersham but using 100 μg C. trachomatis L1/440/LN, 500 μCi (18.5 MBq) Bolton and Hunter reagent in 100 μl PBS adjusted to pH 8.2.

Immunoblotting. Chlamydial antigens separated by SDS-PAGE were equilibrated for 1 h in 4 M-urea, 50 mM-NaCl, 2 mM-Na₂EDTA, 0.1 mM-dithiothreitol in 10 mM-Tris/HCl, pH 7.4. This procedure is reported to improve the antigenicity of polypeptides separated on SDS-PAGE (Bowen et al., 1980). Antigens were electroblotted onto nitrocellulose (0-45 μm; Schleicher & Schuell) at 50 V for 4 h at 4°C in 20 mM-Tris, 150 mM-glycine and 20% (v/v) methanol in water. Molecular mass markers transferred to nitrocellulose were stained by the procedure of Dubois & Rosen (1983), which prevents alteration in the size of the membrane, facilitating comparison with the parent gel. Non-specific binding sites in the membrane were blocked overnight at 4°C in 3% (w/v) BSA (Sigma, Fraction V, pH 7), 0-9% (w/v) NaCl, 0-05% (w/v) NaΝΟ₃ in 10 mM-Tris/HCl, pH 7-4. After washing in gelatin-NP40 buffer (Towbin et al., 1979) the membrane was cut into strips approximately 5 mm wide, and incubated overnight on ice with 250 μl test serum diluted 1 in 10 (trachoma sera) or 1 in 50 in gelatin-NP40 buffer. After washing in gelatin-NP40, strips were incubated for 45 min on ice with 210μl [125I]protein A equivalent to 2 × 10^5 c.p.m. Bolton and Hunter reagent was then determined. Purified C. trachomatis L1/440/ON was radiolabelled at free amino groups with 2-4 μCi Bolton and Hunter reagent and solubilized in Empigen BB. When 5 × 10^5 c.p.m. of Bolton and Hunter reagent was added to 2 μg of C. trachomatis L1/440/ON was radiolabelled at free amino groups with [125I] Bolton and Hunter reagent was added to 2 μg of C. trachomatis L1/440/ON.

RESULTS

Antigenic specificity of LGV sera

Western blotting was used to determine the reactivity of 14 LGV sera with C. trachomatis L1/440/LN. Specificity of the blotting procedure was controlled using 6 human sera negative for chlamydial antibody by both micro-IF and ELISA (Fig. 1a). Control sera showed no false positive reactions with any of the separated chlamydial polypeptides. LGV sera reacted with a wide range of chlamydial polypeptides including a 17 kDa polypeptide, a 29 kDa doublet, a 60 kDa doublet (11/14 sera), a 65 kDa polypeptide (8/14 sera) and polypeptides of approximately 128 and 150 kDa. Antibody to polypeptides of 38 and 41 kDa in the MOMP region of the gel was present in all LGV sera and accounted for around a third of total [125I]protein A binding to the nitrocellulose. Certain sera reacted with both these polypeptides (nos 2, 5, 16 and 18) whilst others reacted preferentially with the upper (nos 3 and 13) or lower (nos 4, 14 and 17) molecular mass polypeptide, confirming that they were immunologically distinct.

The identity of chlamydial surface antigens was confirmed using Western blots of selected sera against chlamydial outer membrane complex purified by the method of Caldwell et al. (1981). Strong antibody responses were observed in the MOMP (40 kDa) region of the gel and to polypeptides with approximate molecular masses of 58 and 63 kDa (Fig. 1b). Weaker antibody responses were detected to polypeptides of 30, 50 and 75 kDa.

The reactivity of the same LGV sera on radioimmunoprecipitation with the same organism was then determined. Purified C. trachomatis L1/440/ON was radiolabelled at free amino groups with [125I] Bolton and Hunter reagent and solubilized in Empigen BB. When 5 × 10^5 c.p.m. of...
Fig. 1. (a) Sera from patients with LGV infection and control negative sera lacking antibody to chlamydiae (*) were reacted by immunoblotting with separated polypeptides of an LGV strain of C. trachomatis (L1/440/LN). Antibody to a range of polypeptides was identified in LGV sera, with MOMP at approximately 40 kDa, a major antigen. Negative control sera did not react with any chlamydial polypeptides, confirming the specificity of the blotting procedures. (b) Chlamydial surface antigens were identified by the Western blot procedure using purified chlamydial outer membrane complex as antigen. The approximate molecular masses (kDa) from SDS-PAGE of the antigens detected are indicated on the right-hand side of this and subsequent figures.
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Fig. 2. Radioimmunoprecipitation assay of antibody to an LGV strain of C. trachomatis in sera from patients with LGV and from negative controls (*). C. trachomatis L1/440/LN was radiolabelled with [\(^{125}\)I]Bolton and Hunter reagent and solubilized in Empigen BB. Sera were incubated with the extract, the antigen–antibody complexes formed were bound to protein A-Sepharose beads, and the radioactive antigen in the complex was identified by SDS-PAGE followed by autoradiography. The dominant antibody response detected was to polypeptides in the MOMP region of the gel.

solubilized antigen was incubated with test serum, 2–3% of the radioactivity became associated with the protein A immunoabsorbent in the case of LGV sera and 0·18% for control sera. The \(^{125}\)I-labelled antigens in the immune complexes were separated by SDS-PAGE and located by autoradiography (Fig. 2).

Antibody to MOMP was found in all LGV sera tested corresponding to 50–88% of the radiolabelled antigen (mean 67%) detected, with typical activity around 250 c.p.m. after background subtraction. At least two antigenic polypeptides were detected in the MOMP region of the gel, but were poorly resolved. Weak antibody to polypeptides of 62 kDa and 29 kDa was detected in 6 and 10 of the 12 sera investigated, accounting for 10% and 14% respectively of the radioactivity due to bound antigen. No significant antibody response was detected in control negative sera.

Cross-reactivity between LGV sera and C. psittaci

To determine whether LGV antibody to C. trachomatis polypeptides would identify genus-specific immunodeterminants on comparable C. psittaci polypeptides, the Western blots were repeated using C. psittaci Cal-10 and GPIC A10 as antigens. C. psittaci MOMP failed to transfer
Antibodies to a wide range of other polypeptides were detected, suggesting that these antigens carry determinants common to both Cal-10 and LGV strains of C. trachomatis. At lower antigen concentrations (not shown) MOMP successfully transferred but did not react with the LGV sera, confirming the lack of genus-specific immunodeterminants.

to nitrocellulose at a loading of 400 μg protein per slab gel but transferred at a loading of 100 μg. LGV antibody failed to react with successfully transferred C. psittaci MOMP of either strain but reacted with a wide range of C. psittaci polypeptides, particularly in the range 58 to 65 kDa (Figs 3 and 4). Strain Cal-10 antigens showed more extensive cross-reactivity with LGV antibody than the GPIC strain, suggesting that this organism is more closely related to LGV strains of C.
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Fig. 4. As for Fig. 3 with *C. psittaci* GPIC A10 as antigen. This strain showed less reactivity with LGV antibody than did *C. psittaci* Cal-10, supporting the concept of considerable antigenic heterogeneity within *C. psittaci*.

trachomatis. Clearly these polypeptides carry genus-specific immunodeterminants common to both chlamydial species. When four sera from patients with psittacosis were reacted on the same gels with *C. psittaci* antigens a similar pattern of reactivity to the LGV sera was observed. No antibody to *C. psittaci* MOMP was detected, suggesting that these organisms may be antigenically heterogeneous, but antibody was again detected to polypeptides in the range 58 to 65 kDa (Figs 3 and 4).

Antigenic specificity of trachoma sera

Sera from trachoma patients from a region with hyperendemic blinding trachoma in southern Iran were characterized by micro-IF. The sera were of low titre and were usually serotype-
Fig. 5. Composite figure based on three comparable gels showing the reactivity by Western blot of trachoma and other immune sera with trachoma antigens: 
*C. trachomatis* SA1, serotype A (a); TW5, serotype B (b, c). Most trachoma sera were type-specific on micro-IF. The type specificity and serum identification number are indicated at the top of the figure. A few trachoma sera showed broader reactivity, with specificity for serotypes A, B and C. Three LGV sera were included for comparative purposes as well as a monoclonal antibody (M) to MOMP and a rabbit hyperimmune serum (R) to *C. trachomatis* L2. Negative control sera confirmed the specificity of the blots but have been omitted for brevity. For a description of the gels see main text.
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specific for trachoma agents of serotype A or B. A few more broadly reactive sera showed specificity for serotypes A, B and C or A and C alone. The antigenic specificity of these sera was then determined using Western blots to C. trachomatis serotypes A (Fig. 5a), B (Figs 5b and 5c) and C (not shown).

Sera repeatedly analysed by Western blot gave remarkably reproducible patterns of reactivity with chlamydial polypeptides as shown by serum 57, the only serum common to all three blots comprising Fig. 5. Thus it was possible to compare Western blots made on separate occasions.

Serotype-specific trachoma sera reacted with a reduced range of polypeptides compared with more broadly cross-reactive trachoma or LGV sera. The sera reacting in Fig. 5a with a serotype A trachoma agent gave virtually identical results when C. trachomatis UW1, serotype C was used as antigen (data not shown). Traces of antibody were detectable in B-specific sera only, to MOMP of serotype A or C (Fig. 5a) and were detected in more significant amounts in broadly cross-reactive sera, including three LGV sera, a rabbit hyperimmune serum to serotype L2 and one (no. 59) of two trachoma sera with reactivity for serotypes A, B and C. An interesting feature of blots with serotype A or C antigen was that all trachoma sera reacted dominantly with a polypeptide of 36 kDa. Antibody to this polypeptide was also present in cross-reactive sera and in blots directed against serotype B antigen (Figs 5b and 5c) but was of less importance. Three serotype B-specific sera were the only trachoma sera showing strong reactivity with the homologous MOMP, the position of which was defined by monoclonal antibody (track M in Fig. 5c). A strong antibody response was also detected to polypeptides of around 63 kDa (e.g. sera 43, 53 and 57 in Figs 5a and 5b).

DISCUSSION

In active trachoma, lymphoid follicles with germinal centres develop in the conjunctiva, giving rise to local, tear sIgA antibody. This antibody is probably important in short-lived immunity after ocular chlamydial infection (Monnickendam & Pearce, 1983) but was not available in adequate amounts for determination of its antigenic specificity. However, migration of immunologically committed plasma blasts from the conjunctival site gives rise to circulating antibody, which was the subject of this study. In man, tear and serum antibody in trachoma is of similar specificity (Treharne et al., 1978). In the guinea pig model of trachoma, both serum and tear antibodies correlate equally well with resistance to ocular challenge (Malaty et al., 1981). Thus the antigenic specificity of serum antibody in trachoma is likely to be similar to that of local tear antibody and can be related to protective immunity.

Micro-IF testing of the sera studied here showed that the trachoma sera were, in general, narrowly serotype specific whereas the LGV sera reacted with a much broader range of the 15 C. trachomatis serotypes. This serological difference was matched by the range of polypeptides with which the sera reacted on Western blotting. Trachoma sera were characterized by a weak immune response to MOMP, despite the presence of an important serotype-specific determinant on this protein, but a strong reaction with a 36 kDa polypeptide, and the virtual absence of reactivity to polypeptides in the range 43 to 58 kDa. By contrast, in LGV sera the dominant immune response was to MOMP. Could the lack of an immune response to critical determinants on MOMP be an explanation for the susceptibility of children in hyperendemic areas to repeated bouts of damaging trachoma infection? To answer this it is necessary to consider the effects which immunoblotting might have on chlamydial antigens.

Chlamydial surface proteins are acidic and heavily cross-linked by S-S bridges. Thus complete solubilization of chlamydial EB, necessary for immunoblotting, requires strongly anionic, denaturing detergents like SDS, together with a reducing agent such as mercaptoethanol (Hatch et al., 1981; Bavoil et al., 1984). Such procedures are incompatible with the preservation of secondary and tertiary protein structure, which must be maintained if conformation-dependent epitopes are to be retained. Thus the major reason for the failure of monoclonal antibodies to human IgG to react on Western blotting was the disruption of secondary structure by mercaptoethanol (Thorpe et al., 1984). Antibodies in broadly cross-reactive sera like the LGV sera binding to conformation-independent determinants would mask
the destruction of conformation-dependent epitopes on Western blotting unless the latter were immunodominant. As MOMP failed to blot under non-reducing conditions it was necessary to renature the separated antigens by removing the SDS with 6 M-urea before electrophoretic transfer (Bowen et al., 1980). This method had beneficial effects on chlamydial antigenicity because two of five monoclonal antibodies with specificity for the MOMP of *C. trachomatis* serotypes B, D, E, L1 and L2 failed to react in Western blots unless it was used (data not shown). Nevertheless, these urea-treated antigens should not be regarded as equivalent to the native proteins.

The simplest explanation for the lack of antibody to MOMP is that the serotype-specific immunodeterminant on MOMP recognized by trachoma sera is conformation-dependent and thus destroyed by Western blotting. This does not explain why certain serotype B-specific trachoma sera showed strong reactivity for MOMP of homologous serotype and weak reactivity with MOMP of serotypes A and C. Perhaps the serotype B-specific determinant on MOMP is more stable than its counterpart on serotypes A or C. Nevertheless, the lack of antibody response in the trachoma patients studied here to the SDS-stable immunodeterminants on MOMP is of interest because it might be associated with their evident lack of immunity to recurrent infection. In support of this concept, antibody to MOMP is known to neutralize chlamydial replication *in vitro* (Caldwell & Perry, 1982), probably by blocking the movement of nutrients through the porin formed by this protein (Bavoil et al., 1984). The critical immunodeterminants involved have not been identified.

In LGV sera antibody to MOMP failed to react with *C. psittaci*, confirming the species specificity of MOMP. This antibody was directed against two closely adjacent but immunologically distinct polypeptides with a difference of approximately 2 kDa (Salari & Ward, 1981). It is unclear whether the lower molecular mass polypeptide in this doublet was unrelated to MOMP or was a distinct form of the protein resulting either from incomplete reduction of the S-S bridged oligomers described by Newhall & Jones (1983) or from incomplete processing leaving the membrane insertion signal peptide still attached. Translation *in vitro* of a 15 kbp insert of chlamydial genomic DNA in *Escherichia coli* yielded two similar, immunologically related bands identified as MOMP (Allan et al., 1984).

Western blotting of LGV sera against purified chlamydial outer membrane revealed, in addition to MOMP, surface polypeptides of approximately 19, 30, 49, 58, 63 and 74 kDa. The 19 and 74 kDa polypeptides may be identical to the chlamydial surface polypeptides of 17 and 74 kDa which have recently been cloned (Wenman & Lovett, 1982a; Kaul & Wenman, 1985). If so, this study suggests that these surface polypeptides were quantitatively of minor importance in the immune response to LGV (Fig. 1a). The 30 kDa surface polypeptide may be equivalent to the 29 kDa polypeptide described by Newhall et al. (1982). The minor surface antigen of approximately 49 kDa has not previously been described. The surface polypeptides between 58 and 65 kDa, antibody to which was genus-specific and occurred in most chlamydial infected patients, probably overlap with the polypeptides of 60–62 kDa which Newhall et al. (1982) found in the sera of patients with chlamydial genital tract infection. Antibody to LPS would not have been detected in this study due to its greater electrophoretic mobility and its failure to bind to nitrocellulose in the presence of detergent.

A major point of difference between this study and that of Newhall et al. (1982) lies in the reactivity of chlamydial polypeptides with control sera. Newhall and colleagues detected antibody to MOMP and the 29 kDa polypeptide in all control sera from subjects presumed, on epidemiological grounds, not to have experienced chlamydial infection. Low levels of antibody to porins on other bacteria might cross-react with chlamydial MOMP given the conserved nature of the amino acid sequences of porins (Hofstra & Dankert, 1979) at least amongst *Enterobacteriaceae* species. If so, these antibodies must be of low avidity. In this study conditions of Western blotting were stringently established so that antibodies in immune sera were readily detected, whereas no non-specific immunoglobulin binding was detected in the sera of control subjects selected for their absence of chlamydial antibody (see Fig. 1). In 20 randomly selected blood donor sera traces of antibody to the polypeptide of 62 kDa were detected in three sera only,
of which two sera had chlamydial antibody detectable by ELISA. No antibody was detected to MOMP or the polypeptide of 29 kDa.

A difficulty is that many of the chlamydial polypeptides antigenic in man identified by this study are of unknown function. Once information on this becomes available the importance of specific immune responses identified here should become more apparent. The techniques used here are being applied in conjunction with clinical and epidemiological observation of trachoma in an African community to determine any protective role in man of antibody to defined chlamydial antigens and whether such antibody plays a role in the immunopathology of blinding trachoma.

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REFERENCES


