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Identification of Genus-Specific Epitopes on the Outer Membrane Complexes of *Chlamydia trachomatis* and *Chlamydia psittaci* Immunotypes 1 and 2

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Polyclonal and monoclonal antibodies were used to study the immunogenic and antigenic characteristics of chlamydiae. We focused on the most predominant proteins in the outer membrane complex, the major outer membrane protein (MOMP) and the doublet consisting of proteins of 57 and 62 kilodaltons (57–62 kDa doublet). Immunoblot analyses were performed with chlamydial elementary bodies by using (i) immune sera from sheep which had undergone a recent episode of abortion due to the ovine abortion (OA) strain of *C. psittaci*, (ii) rabbit hyperimmune anti-*C. psittaci* (OA) and -*C. trachomatis* sera, and (iii) monoclonal antibodies to the MOMP of *C. trachomatis*. The typical pattern of response with polyclonal antisera against heterologous elementary bodies was reactivity with the 57–62 kDa doublet and lipopolysaccharide with weak and sometimes no anti-MOMP activity. Three distinct genus-specific anti-*C. trachomatis* MOMP monoclonal antibodies showed different patterns of reactivity with the MOMPs of the two immunotypes of *C. psittaci* and *C. trachomatis* serovars. Our data confirm the predominance of a genus-specific 57–62 kDa doublet response despite the presence of genus-specific epitopes on the MOMP.

Chlamydiae are procaryotic obligate intracellular parasites of eucaryotic cells (8, 27, 36) for which extracellular replication has not been demonstrated. They are characterized by a unique developmental cycle which serves as the basis for the current taxonomic classification. Infection is initiated by adhesion to a susceptible cell and a chlamydia-specified phagocytosis (12, 46) of at least one infectious elementary body (EB). In this phagosome, the EB (200 to 300 nm) undergoes differentiation into a larger metabolically active form, the reticulate body (800 to 1,000 nm), that divides by binary fission. The cycle is completed by the reorganization of reticulate bodies into EBs with release of the latter from the infected host cell into the surrounding environment, where more susceptible cells may be infected. The inner and outer double-membrane system, a characteristic feature of gram-negative bacteria, surrounds chlamydial cells at all stages of development. The chlamydial cell envelope, however, appears to lack a peptidoglycan layer in the periplasm (5, 14). The ability of this organism to enter various types of cells, evade certain host cell responses, and participate in the pathogenic and immunopathological aspects of diseases has been attributed to the mosaic of macromolecules which constitute the cell envelope. For this reason, most recent studies on chlamydiae have been directed to this structure. The major outer membrane protein (MOMP) (38 to 40 kilodaltons [kDa]) (22, 35) is a cysteine-rich macromolecule which forms disulfide-linked complexes in the outer membrane (21, 29). The structural and functional roles of this protein have been described (7, 20). Two other extraordinarily cysteine-rich structural proteins having apparent molecular masses (MMs) of 57 to 62 kDa (the 57–62 kDa doublet) and 12 kDa are also present (6).

Human and avian chlamydial infections have been the focus of intensive study in recent years. *Chlamydia psittaci* infections, however, occur in a variety of other animals (26), and reviews of many results of these infections (such as pneumonia, abortions, enteritis, polyarthritis, encephalitis, and conjunctivitis) have been published (37).

Stamp et al. first identified *C. psittaci* as the cause of enzootic abortion of ewes (38). Ewes which have experienced chlamydial abortion acquire a protective immunity and subsequently maintain viable pregnancies (1). It is important that this ruminant pathogen has also been implicated as a cause of human abortions (9, 41).

Ruminant abortion and polyarthritis strains, classified as immunotypes 1 and 2, respectively (32), and representative *C. trachomatis* serovars were used in this study with the objective of identifying any genus- or species-specific epitopes on the outer membrane complexes (OMC) of these organisms.

C. psittaci B577 (immunotype 1) and *C. psittaci* IPA (immunotype 2), originally isolated from the kidney of an aborted ovine fetus and the joint fluid of a sheep, respectively, were obtained from the American Type Culture Collection, Rockville, Md. Two other *C. psittaci* ovine abortion (OA) isolates, V286 and V287, were originally isolated in 1988 from aborted caprine and ovine fetuses, respectively, by P. Menzies, University of Guelph, Guelph, Ontario, and passaged in the yolk sacs of 7-day-old embryonated hens' eggs by D. Key, Ontario Ministry of Agriculture and Food, Guelph, Ontario. *C. trachomatis* serotypes A, Ba, C, D, E, F, G, I, J, K, L_1, and L_2 were also obtained from the American Type Culture Collection. All organisms were grown in HeLa 229 cell monolayers (31) in 175-cm^2 polystyrene culture flasks (Lux; Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.) in 50 ml of Eagle minimum essential medium supplemented with 10% fetal bovine serum, 100 μg of vancomycin hydrochloride per ml, 2 mM L-glutamine, 25 U of nystatin per ml, and 0.5 μg of cycloheximide per ml. The purification method followed was essentially that described previously (31). For the *C. psittaci* EBs, both the infected-cell monolayers and the medium

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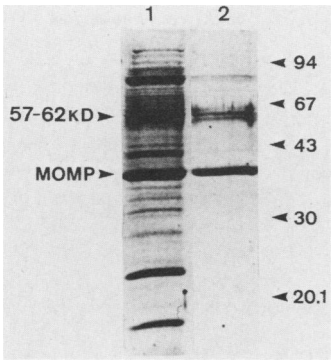

FIG. 1. Coomassie blue-stained SDS-PAGE gel of *C. psittaci* (B577) and the components derived after Sarkosyl extraction. Lane 1 shows the profile of intact EBs, and lane 2 shows the pellet obtained after Sarkosyl extraction. MM markers in kilodaltons (kD) are represented. Arrowheads indicate the positions of the low-MW marker proteins (MWs are given in thousands) phosphorylase b (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), and trypsin inhibitor (20,100).

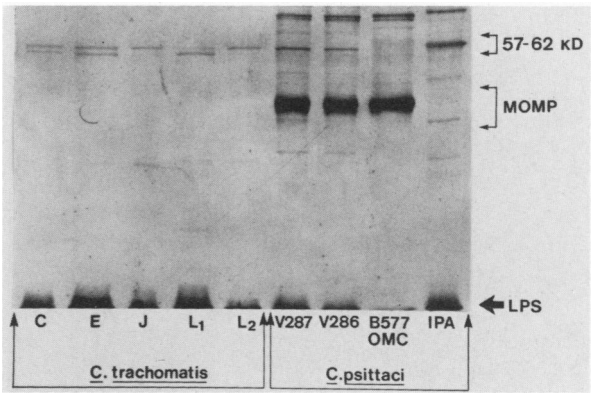

FIG. 2. Immunoblots of *C. psittaci* (V286, V287, and B577 OMC) and *C. trachomatis* (serovars C, E, J, L_1, and L_2) after separation by SDS-PAGE. Immune serum (1/1,000) from a sheep which had experienced a recent episode of *C. psittaci* OA-mediated abortion was used for this analysis. The positions of the 57–62 kDa doublet, MOMP, and LPS are indicated. The immune sera were unreactive with the MOMPs of heterologous EBs and did not recognize the 57–62 kDa proteins in the OMC preparation of *C. psittaci* OA. Sera from seven other sheep that had also undergone abortions gave identical results.

containing free organisms were collected for isolation of the organisms.

Proteins of *Chlamydia* spp. were electrophoretically resolved on 12.5% acrylamide slab gels as described previously (14, 24), with a 0.75-mm gel thickness. Gels were stained with Coomassie brilliant blue and/or silver stain (Bio-Rad Laboratories, Richmond, Calif.) (results not shown). The MM of the MOMP of the three ruminant OA isolates was approximately 37 kDa, which was lower than that of the MOMP of *C. trachomatis* serovars. Isolation of the OMC was performed by using a modification of a procedure previously described (14). Briefly, 1 mg of *C. psittaci* (2 ml) was suspended in 19 ml of 50 mM Tris hydrochloride buffer (pH 8.0) containing 10 mM disodium EDTA and 0.15 M NaCl. Disodium *N*-lauroylsarcosine (Sarkosyl; Sigma Chemical Co., St. Louis, Mo.) was added to a final concentration of 2%. The mixture was vortexed and incubated at 65°C for 1 h. The Sarkosyl-insoluble fraction containing the chlamydial OMC was pelleted by centrifugation at 80,000 × *g* for 1 h at 4°C. The pellet was washed with the suspension buffer and suspended in 2 ml of buffer. The Sarkosyl-insoluble outer membrane preparation contained the MOMP, the 57–62 kDa doublet, and a 75-kDa protein (Fig. 1).

A Transblot apparatus (Bio-Rad Laboratories, Richmond, Calif.) was used for immunoblots (42). Typically, transfer was achieved at 100 mA for 16 to 18 h. The antibody dilution buffer consisted of phosphate-buffered saline containing 0.05% Tween 20 and 1.5% Carnation nonfat skim milk. Immunoblots were developed with peroxidase-conjugated rabbit anti-sheep immunoglobulin G heavy and light chains [IgG(H+L)], goat anti-mouse IgG(H+L) (Organon Teknika, Malvern, Pa.), and goat anti-rabbit IgG(H+L) (Jackson Immunoresearch Labs Inc., Westgrove, Pa.).

First, immunoblots were performed with sera from eight sheep which had experienced a recent episode of *C. psittaci*-mediated abortion (Fig. 2) to identify genus-specific epitopes on *C. trachomatis*. Sera from all eight sheep contained antibodies which recognized the 57–62 kDa doublet and lipopolysaccharide (LPS) of *C. trachomatis* with no anti-*C. trachomatis* MOMP reactivity. Immune ovine sera reacted in a similar fashion with *C. psittaci* IPA. Interestingly, no reactivity with the 57–62 kDa proteins was obtained when the Sarkosyl-insoluble OMC preparation of *C. psittaci* was probed. This may reflect antigenic modification due to the extraction procedure.

To further investigate this observation obtained with naturally infected sheep, the resolved polypeptides of *C. trachomatis* were probed with rabbit anti-*C. psittaci* OA sera. Polyclonal rabbit antisera against *C. psittaci* OA EBs were produced by the immunization of female New Zealand White rabbits with 500 μg of purified EBs mixed with Freund complete adjuvant. Animals were immunized by intramuscular injections in both hind legs and subcutaneously at random sites weekly for the first 3 weeks and then every 10 days for a period of 60 days. The reactivity pattern was again very similar to that obtained in the previous experiments. Intense bands of the 57–62 kDa doublet were revealed with all the *C. trachomatis* serovars tested, but for the *C. psittaci* strains only one band of the doublet was evident (results not shown). There was no demonstrable anti-MOMP response for the *C. psittaci* resolved denatured EBs and only a weak response to the MOMP of *C. trachomatis*. The common immunodominant aspects of chlamydiae were further verified when serum from a rabbit immunized with 50 μg of *C. trachomatis* serovars A, C, D, H, K, and L_2 recognized the major polypeptides of representative serovars of denatured EBs of that species but reacted primarily with one of the polypeptides in the 57–62 kDa doublet and with the LPS of *C. psittaci* OA and IPA isolates (Fig. 3).

We also examined the immunoblot reactivity of murine anti-*C. trachomatis* antibodies against the resolved polypeptides of *C. psittaci* OA and IPA EBs. Polyclonal murine *C. trachomatis* antisera against whole EBs were produced by immunizing BALB/c mice intraperitoneally every 2 weeks with 100 μg of EBs from serovars A, D, and H of *C. trachomatis*. Reactions with both the MOMP and 57–62 kDa doublet of *C. psittaci* abortion isolates were obtained. In contrast to the immunoblots performed with the rabbit and sheep sera, the 57–62 kDa doublet recognized by the mouse serum was resolved in a very diffused band (results not shown).

In most cases, the anti-LPS activity was evident. This was

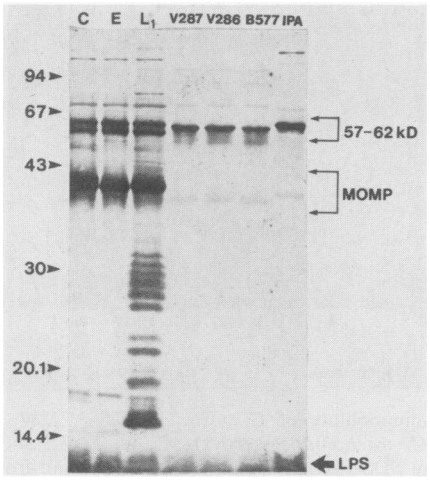

FIG. 3. Immunoblots of *C. psittaci* (V287, V286, B577, and IPA) and *C. trachomatis* (C, E, and L_1) after separation by SDS-PAGE. Serum (1/1,000) from a rabbit immunized with *C. trachomatis* serovars A, C, D, H, K, and L_2 was used in this analysis. MM markers in kilodaltons are on the left. Note the predominance of a cross-reactive response for both the LPS and 57–62 kDa doublet. Only one band of the latter appears to be recognized for the *C. psittaci* strains.

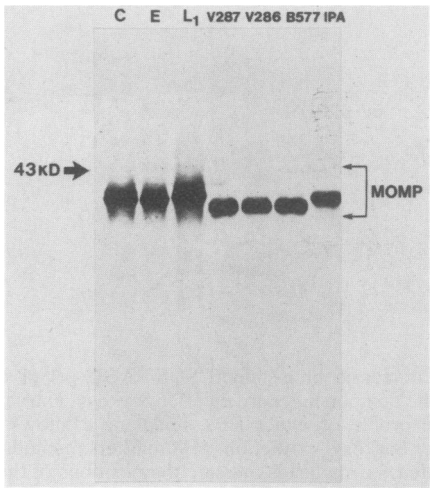

FIG. 4. Example of an immunoblot developed with MAb 102 reactive with *C. trachomatis* (C, E, and L_1) and *C. psittaci* isolates after separation by SDS-PAGE. The position of the MM marker ovalbumin is indicated in kilodaltons (kD) on the left.

supported by the fact that proteinase K digestion of EBs followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis with anti-MOMP monoclonal antibodies (MAbs), anti-chlamydial LPS MAbs, and polyclonal anti-*Chlamydia* spp. sera revealed extensive or complete proteolysis of various resolved proteins, leaving the LPS unaffected.

Two of the three MAbs to *C. trachomatis* MOMP discussed in this paper (597 and 102) were prepared by the standard method (23) using the fusion of X63-Ag 8653 myeloma cells with splenocytes obtained from BALB/c mice immunized intraperitoneally each week for a total of 6 weeks with 2 to 5 μg of OMC preparations derived from serovars C and D in Freund complete adjuvant. Fusions were performed 3 days after the last immunization. The immunogen used for the generation of MAb 271 was a recombinant peptide containing the entire variable domain IV sequence of serovar D MOMP.

Two of the MAbs (597 and 102) which reacted with *C. trachomatis* MOMP by immunoblot analysis also reacted with the MOMP of *C. psittaci* isolates, suggesting recognition of genus-specific epitopes on the MOMP of *C. psittaci*. MAb 271, however, reacted in a species-specific fashion and only recognized *C. trachomatis* serovars. Figure 4 shows the pattern obtained with MAb 102. In order to determine whether the MAbs used in the immunoblot analyses recognized immunoaccessible epitopes of MOMP, indirect enzyme-linked immunosorbent assays (ELISAs) were performed (Table 1). Polystyrene microtiter well strips (Nunc, Roskilde, Denmark) were coated with 5 μg of purified EBs of *C. trachomatis* and *C. psittaci* per ml in 0.05 M sodium carbonate buffer, pH 9.6. Monoclonal anti-MOMP IgG antibodies purified on Protein A (Pharmacia Fine Chemicals, Piscataway, N.J.) were serially diluted from 100 μg/ml to 1 μg/ml with phosphate-buffered saline–0.05% Tween 20 containing 0.25% bovine serum albumin. The assay was done as previously described (45). MAb 597 consistently generated the highest absorbance values at each concentration of antibody. These results were thus chosen as reference absorbance values, and all other readings were expressed as a percentage of the mean of these values. MAb 597, which was immunoblot positive for both species, recognized all *C. trachomatis* serovars and the two immunotypes of *C. psittaci* in the indirect ELISA. MAb 271, which was only reactive with *C. trachomatis* MOMP by immunoblot analysis, recognized the intact EBs of *C. psittaci* OA and *C. psittaci* IPA in the indirect ELISA procedure. MAb 102, which, like MAb 597, reacted strongly with the MOMP of both species in the immunoblot, failed to recognize intact EBs of any of the *C. trachomatis* serovars or of the two *C. psittaci* immunotypes even when used at 100× the working dilution. Table 1 summarizes the combined results obtained by the indirect ELISA procedure and immunoblot analyses.

Previous studies for *C. psittaci* immunological heterogeneity by the plaque reduction technique (33, 34) and immunofluorescence (2, 16, 32, 39) did not define the components on the outer membrane responsible for the differences observed. Furthermore, few attempts to compare the antigenic structure of this species with that of *C. trachomatis* have been made. Our experiments have revealed antigenic homology among *C. trachomatis* and *C. psittaci* ruminant isolates. The characteristic feature of the immune response in sheep was the reactivity with the 57–62 kDa doublet in the heterologous *C. trachomatis* EBs. Evidence for the genus-specific immunodominant nature of this doublet was also reflected in the immunoblots using polyclonal rabbit and murine antisera

TABLE 1. Reactivity patterns of MOMP MAbs

MOMP MAb	Antibody reactivity with:					
	C. trachomatis serovar L_1		*C. psittaci* OA		*C. psittaci* IPA	
	ELISA[a]	Immunoblot	ELISA	Immunoblot	ELISA	Immunoblot
597	100	+	100	+	70	+
271	80	+	40	−	20	−
102	0	+	0	+	0	+

[a] Results represent percent absorbance values (A_{492}) and reflect the accessibility of the MOMP epitope to the MAbs.

reacted against heterologous EBs. It is not clear why the MOMP of *C. psittaci* OA, although the most abundant protein in the outer membrane, failed to be as immunogenic as the 57–62 kDa doublet in the animals studied despite the presence of genus-specific epitopes on the MOMP. Although other workers have described the predominance of an anti-57–62 kDa response in active human *C. trachomatis* infection (10, 11, 28), we have shown that the anti-57–62 kDa response, along with the anti-LPS response, is primarily genus specific and predominates over the anti-MOMP response in naturally or experimentally infected animals. The predominance of this genus-specific response to the 57–62 kDa doublet may be a reflection of a high degree of polypeptide homology in this protein between the two chlamydial species or the presence of one or more exposed critical conserved immunodominant residues. This may also reflect optimum presentation of the 57–62 kDa proteins following antigen processing by an antigen-presenting cell. An early response to an outer membrane protein of chlamydiae may play a role in facilitating early entry into some susceptible cells by opsonization. Conversely, a response to these proteins may limit the spread of the organisms by blockade of a possible adhesin located on the 57–62 kDa doublet. The establishment of antibody response to this doublet may also have some diagnostic value as a marker of early chlamydial infection. Allen and Stephens (2) have recently suggested that the computer-predicted high frequency of β turns on the 60-kDa protein of *C. trachomatis* L_2 may in part explain the intensity of the immune response to this extraordinarily cysteine-rich protein.

Murine polyclonal antibodies to *C. trachomatis* were more reactive with the abortion isolates of *C. psittaci* than with the IPA strain, reflecting a closer relationship of *C. psittaci* immunotype 1 to *C. trachomatis* or the antigenic modification of the outer membrane of *C. psittaci* IPA under these experimental conditions. The three different MAbs to *C. trachomatis* MOMP which also reacted with the two *C. psittaci* immunotypes targeted genus-specific epitopes on the MOMP of these isolates, and qualitative and quantitative differences were noted in the reactivities of these antibodies. The fact that MAb 271 failed to recognize the MOMP of *C. psittaci* in immunoblots but reacted with both immunotypes in the ELISA suggests that the chlamydial antigenic epitope on *C. psittaci* recognized by this MAb is conformational and was destroyed in SDS-PAGE. MAb 102 probably recognizes a membrane-embedded genus-specific epitope of chlamydia MOMP, since it reacted very strongly in immunoblots but failed to react in ELISA with any of the *C. trachomatis* serovars or *C. psittaci* immunotypes. It is also possible that the interaction of intact EBs with polystyrene resulted in the modification of the epitope recognized by the MAb 102. Immunological typing of *C. psittaci* with MAbs reactive with exposed structures of this species (3, 18) and with MAbs which recognize LPS and MOMPs of various *C. psittaci* isolates (17) has been attempted, although a role for these outer membrane components in pathogenesis has yet to be investigated for *C. psittaci*. The significance of the MOMP of *C. trachomatis* has been already deduced from neutralization studies with polyclonal anti-MOMP (15) and monoclonal anti-MOMP (25, 31, 47) antibodies. The anti-LPS activity noted in immune sheep, rabbit, and mouse polyclonal sera represents the recognition of genus-specific epitopes previously described for chlamydiae (13, 30).

Although in the study of human infections etiological inferences are best drawn from subhuman primates (40), in the case of *C. psittaci* ovine abortion isolates, their capacity to cause abortion in both humans and ruminants and their antigenic similarity to *C. trachomatis* signify that studies in both types of hosts may contribute to the understanding of the disease in either. It is particularly important for future studies to establish more reliable classification criteria for the nonavian stains of *C. psittaci*. Clearly, the microimmunofluorescence test of Wang and Grayston (19, 43), which resulted in the classification of *C. trachomatis* isolates in 15 distinct serovars, proved to be very useful (19, 44), and a correlation between serotype and pathogenic spectrum was established (35). In contrast, the typing of *C. psittaci* is in a very early stage and thus requires urgent attention for the complete understanding of chlamydial epidemiology and zoonotic potential.

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