Monoclonal Antibody Against a Genus-Specific Antigen of Chlamydia Species: Location of the Epitope on Chlamydial Lipopolysaccharide

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Monoclonal antibodies were prepared by the fusion of murine myeloma NS1 cells with spleen cells of BALB/c mice immunized with Formalin-killed elementary bodies of the Chlamydia trachomatis L2 serovar. The specificity of these monoclonal antibodies was determined with a solid-phase immunosay in which HeLa 229 cells infected with C. trachomatis serovars D, G, H, I, L2 and the Chlamydia psittaci meningopneumonitis strain Cal-10 were used. An immunoglobulin G monoclonal antibody (L2I-6) was identified that reacted with both C. trachomatis- and C. psittaci-infected HeLa cells. The immunoreactivity of the genus-specific epitope was heat resistant (100°C, 10 min) but was destroyed by sodium metaphosphate treatment. Further characterization of the chlamydial specificity of monoclonal antibody L2I-6 by microimmunofluorescence showed that it was reactive with all 15 C. trachomatis serovars and seven C. psittaci strains isolated from five different animal species. We undertook studies to identify the biochemical nature of the chlamydial component on which the genus-specific epitope was located. The immunoreactive component was isolated by hot phenol-water extraction of dithiothreitol-reduced chlamydial elementary bodies. The component was positive in the Limulus amoebocyte lysate test (results of Limulus amoebocyte lysate assay were identical with those of Salmonella typhimurium LT2 SAI 377 Re lipopolysaccharide [LPS]), contained 8.8% 2-keto-3-deoxyoctulosonic acid, was resistant to proteinase K, and possessed electrophoretic mobility and silver-staining characteristics in sodium dodecyl sulfate-polyacrylamide gel electrophoresis consistent with a rough LPS or glycolipid. On the basis of these findings, we conclude that the genus-specific epitope recognized by monoclonal L2I-6 is located on chlamydial LPS. We further characterized the antigenic properties of the chlamydial LPS epitope by examining the immunoreactivity of monoclonal antibody L2I-6 by immunoblotting analyses against isolated LPSs extracted from Neisseria gonorrhoeae, S. typhimurium, and Escherichia coli. Monoclonal antibody L2I-6 did not bind LPS of these organisms, demonstrating that the chlamydial genus-specific LPS epitope is apparently not shared by these gram-negative bacteria. We were able, however, to show that the chlamydial LPS does share antigenic determinants with LPSs of gram-negative organisms. Polyclonal rabbit antisera raised against S. typhimurium Re LPS or lipid A showed intense immunological cross-reactivity with chlamydial LPS by immunoblotting. The anti-S. typhimurium Re LPS serum reacted with both C. trachomatis and C. psittaci LPS and its homologous Re LPS but failed to bind to LPS of the Ra chemotype or smooth LPS. In contrast, rabbit anti-S. typhimurium lipid A serum recognized LPSs of both chlamydial species, all three S. typhimurium chemotypes (smooth, Ra, and Re), and N. gonorrhoeae strain JS1. These immunological findings indicate that the chlamydial LPS on which the chlamydial genus-specific epitope resides is structurally similar to the Re LPS of S. typhimurium. These observations also show that the LPS of Chlamydia spp. consists of at least three antigen domains, two of which are shared by the LPS of certain gram-negative organisms and another that is unique to chlamydial LPS.

All members of the genus Chlamydia share a common group antigen characterized by its resistance to heating and sensitivity to oxidation by sodium periodate (28). These early observations have led investigators to suggest that the group antigen is carbohydrate in nature (3, 5). Dhir et al. (12) were the first to isolate this antigen and partially characterize its biochemical nature. Their studies showed that the antigen was a glycolipid moiety and that the group antigen determinant resided in the polysaccharide portion of the molecule. In subsequent studies, these investigators reported that mild acid hydrolysis of the isolated polysaccharide antigen separated the molecule into acidic and neutral constituents, both of which retained the chlamydial group antigen activity (13). The isolated acid polysaccharide antigen gives a positive thiobarbituric acid test and behaves very similarly, but not identically, to 2-keto-3-deoxyoctulosonic acid (KDO) isolated from Salmonella typhimurium by paper chromatography. These observations led these investigators to suggest that the group antigen was located on a structure similar to the KDO moiety of Salmonella spp. lipopolysaccharide (LPS). Comparative antigenic analyses of the isolated chlamydial group antigen polysaccharide and Salmonella LPS or KDO were not reported; nevertheless, these initial observations were the first suggestions that the chlamydial group antigen may have structural properties similar to those of the LPS of gram-negative bacteria.

Recently, Nurminen et al. (24) isolated the chlamydial group glycolipid antigen from elementary bodies (EBs) by the phenol-chloroform-petroleum ether (PCP) LPS extraction method of Galanos et al. (16). These investigators have presented immunological evidence showing that the isolated

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glycolipid antigen is antigenically related to Salmonella LPS. By enzyme immunoassay, rabbit antisera raised against the Re chemotype mutant (LPS composed of KDO and lipid A only) of S. typhimurium or Salmonella minnesota cross-reacts strongly with isolated chlamydial glycolipid antigen. These immunological findings, together with the molecular sizes, solubility, and endotoxic properties of the glycolipids, led these investigators to propose that the chlamydial glycolipid antigen is an LPS-like molecule which closely resembles the Re LPS of Salmonella spp.

In this report, we confirm the findings of Nurminen et al. (24) that Chlamydia spp. possess LPS which shares antigenic determinants with the Re LPS of Salmonella spp. However, in addition, we describe a monoclonal antibody that recognizes an immunodominant epitope that is located on chlamydial LPS, is unique to Chlamydia spp., and is not present on the LPS of other gram-negative bacteria.

**MATERIALS AND METHODS**

**Organisms.** Chlamydia trachomatis was grown in HeLa 229 cells or suspension cultures of mouse L-929 cells, and EBs were purified as previously described (8). Neisseria gonorrhoeae JS1 was grown on Wong-Shockley-Johnston medium (34). S. typhimurium LT2 strains SGSC 228, SGSC 229, SGSC 230, and SA1 377 were grown in trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.). These strains were obtained from K. Sanderson, Salamonella Genetic Stock Center, Calgary, Alberta, Canada.

**Antisera.** Hyperimmune rabbit polyclonal antisera against purified EBs were prepared as previously described (10). The immunoglobulin G (IgG) fraction of the antisera was isolated by adsorption to protein A-Sepharose CL-4B (Sigma Chemical Co., St. Louis, Mo.) as described by Goding (17). Rabbit polyclonal antisera against purified S. typhimurium LT2 G30/C21 Re LPS and lipid A were kindly provided by Charles McLaughlin, Laboratory of Microbial Structure and Function, Hamilton, Mont.

**Hybridoma production.** Female BALB/c mice (3 to 4 weeks of age) were immunized with 4.0 × 10⁶ inclusion-forming units of Formalin-killed L2 EBs mixed with 0.1 ml of Freund complete adjuvant (Difco Laboratories, Detroit, Mich.). Approximately 0.1 ml of adjuvant-EB emulsion was administered intramuscularly in a hind leg of each mouse on days 0, 7, 14, and 21, and 4 × 10⁸ inclusion-forming units of Formalin-killed L2 serovar was injected intravenously. On day 31, mice were sacrificed and their spleens were removed. Hybridomas of BALB/c spleen cells and murine myeloma cells (P3-NS-1-Ag-4/1) were prepared as described by Barbour et al. (2).

**Selection and cloning hybridomas.** Supernatants from wells containing growing hybridomas were assayed by a solid-phase radioimmunoassay in which chlamydial-infected HeLa 229 cells were used. Briefly, 5 × 10⁴ HeLa 229 cells suspended in 100 μl of Eagle minimal essential medium in Hanks balanced salts and supplemented with 10% fetal bovine serum were seeded into wells of a 96-well, flat-bottomed tissue culture tray (Linbro, Flow Laboratories, Inc., McLean, Va.). After 24 h, the medium was removed and six rows of 12 wells were infected with Chlamydia psittaci Mn and C. trachomatis strains L2/434/Bu, D/UV-3/Cx, G/UW-57/Cx, H/UW-4/Cx, and I/UW-12/Ur. A single row of wells was not infected and served as a HeLa cell control. At 42 h postinfection, the medium was removed from wells containing cells infected with the Mn strain and the L2 serovar. These monolayers were then fixed with 1 ml of absolute methanol for 10 min and washed twice with 1 ml of 20 mM NaPO₄-0.15 M NaCl-0.02% NaN₃, pH 7.2 (phosphate-buffered saline [PBS]-azide). One milliliter of PBS-azide was added to wells containing fixed cells, and the trays were then incubated at 37°C in 5% CO₂ for an additional 24 h. At this time, the remaining monolayers (non-LGV strains and uninfected control cells) were fixed and processed as described above.

For screening of chlamydial antibody, supernatant fluid (100 μl) from wells containing proliferating hybridomas was inoculated onto fixed HeLa monolayers that were infected with the different chlamydial strains and incubated for 2 h at 37°C on an orbital shaker (Bellco Glass Inc., Vineland, N.J.). The fluid was then removed, and each well was washed three times with PBS-azide for 10 min per wash. To each well 50 μl of ¹²⁵I-labeled protein A (5 × 10⁴ cpm) was added, and the plates were reincubated at room temperature for 2 h on an orbital shaker. Finally, the iodinated protein A solution was removed, and the wells were washed as described above. The plate was then subjected to autoradiography with Kodak X-Omat AR film and a Lightning-Plus intensifying screen for 12 to 18 h at −68°C. Wells demonstrating bound protein A were identified, and the respective supernatant fluids were reassayed by immunoblotting to identify the immunoreactive component (see below). Hybridomas of interest were then cloned twice by limiting dilution. Monoclonal antibodies were typed by double immunodiffusion with subclass-specific goat anti-mouse IgG antisera (Litton Bionetics, Kensington, Md.).

The effect of periodate treatment on chlamydial antigens was determined by the solid-phase infected-cell assay described above. Methanol-fixed, infected HeLa 229 cell monolayers were treated with 100 μl of 0.05 M sodium metaperiodate in 0.05 M sodium acetate buffer (pH 5.5) or buffer alone at 4°C for 24 h. The wells were washed with PBS-azide and probed with antibody and ¹²⁵I-labeled protein A as described above. Susceptibility to periodate oxidation was considered positive when periodate pretreatment inhibited or greatly reduced monoclonal antibody binding to chlamydia-infected cells as determined by exposure of the autoradiograms.

The microimmunofluorescence method of Wang (32), developed for the serological classification of C. trachomatis, was used to determine the chlamydial specificity of monoclonal antibodies.

**Polyacrylamide gel electrophoresis.** The sodium dodecyl sulfate-polyacrylamide gel electrophoresis procedure of Laemmli (20) was used.

**Isolation of chlamydial LPS.** EBs were harvested from L-929 cells 42 h after infection. Purified EBs were washed twice with PBS, and the pellet was resuspended in glass distilled water and lyophilized. The dry weight of L2 EBs obtained from a typical harvest of 2 × 10⁹ infected L-929 cells was between 60 and 65 mg. EBs (60 mg [dry weight]) were initially extracted by the PCP method of Galanos et al. (16). When we were unable to precipitate LPS from the phenol phase by the addition of water, the PCP residue was reextracted by the hot phenol-water method of Westphal and Jann (33). The procedure was modified in that ultracentrifugation was extended from 3 to 16 h. Subsequently, EBs were reduced with 50 mM dithiothreitol (Sigma) in 20 ml of PBS for 30 min at 4°C. One milliliter of 1.0 M iodoacetamide in PBS was added, and the mixture was kept at 4°C for 1 h. The reduced and alkylated EB suspension was centrifuged at 30,000 × g for 15 min at 4°C, and the EB pellet was resuspended in 10 ml of cold distilled water. This suspension was then lyophilized and weighed. The LPS from 32.6 mg
(dry weight) of EBs treated in this manner was readily extracted by the hot phenol-water method. The length of ultracentrifugation was again increased to 16 h.

Isolation of gonococcal and salmonella LPSs. N. gonorrhoeae LPS was isolated by the procedure of Westphal and Jann (33) as modified by Perry et al. (26). The LPS of S. typhimurium LT2 smooth chemotype was isolated by the procedure of Westphal and Jann (33). The LPSs of the Ra, Rd2, and Re chemotypes were extracted by the method of Galanos et al. (16).

Silver staining. The silver-staining method of Tsai and Frasch (31), as modified by Hitchcock and Brown (18), was used. The protease susceptibility of immunoreactive components was determined by treating chlamydial whole-cell lysates with 0.25 mg of proteinase K per ml at 60°C for 18 h. Control samples were treated identically, but without the addition of proteinase K.

Immunoblotting. The immunoblot procedure of Towbin et al. (30) and of Bittner et al. (6) as modified by Batteiger et al. (4) was used. Briefly, electrophoretic transfer was done in 25 mM sodium phosphate (pH 7.3) at 27 V (0.9 to 1.0 A) for 2 h at 17°C to HAHY Millipore nitrocellulose paper with a Trans-Blot cell (Bio-Rad Laboratories, Richmond, Calif.). After transfer, the nitrocellulose paper was incubated in 50 mM sodium phosphate–0.15 M NaCl–0.02% NaN3 containing 3% bovine serum albumin for 30 min. The nitrocellulose paper was then incubated with antiserum or monoclonal antibody diluted in PBS-bovine serum albumin for 2 to 16 h at room temperature. The nitrocellulose paper was then washed in PBS, pressed between sponges, and air dried at 37°C. In our hands, chlamydial LPS bound very poorly to nitrocellulose paper when buffers containing detergents (Tween-20 or Nonidet P-40) were used. We therefore omitted detergent from all immunoblotting procedures. Autoradiography was done with Kodak X-Omat AR film with a Lightning-Plus intensifying screen at −68°C for 4 to 12 h.

RESULTS

Identification and specificity of monoclonal antibodies. Hybridoma supernatants were screened for immunoreactivity to chlamydial antigen(s) with a solid-phase radiometric assay in which were employed HeLa 229 cells infected with the C. trachomatis serovars L2, D, and G (B serogroup) and H and I (C serogroup) and C. psittaci Mn. Hybridoma supernatants were screened in duplicate against chlamydia-infected HeLa cells that had been treated with 0.05 M NaIO4 in 0.05 M acetate buffer or acetate buffer only. Treatment with NaIO4 was included in the initial screening assay to identify protein A-binding monoclonal antibodies that were reactive against the periodate-sensitive antigen(s). The results of a typical assay are shown in Fig. 1. In Fig. 1, lane b, the reactivity of the hybridoma L21-6 supernatant fluid is shown. The L21-6 monoclonal antibody reacted with HeLa cells infected with C. psittaci Mn and C. trachomatis serovars L2, D, G, H, and I, but not the infected HeLa cell control. The immunoreactivity of this group-reactive antibody was destroyed by prior treatment with NaIO4 (Fig. 1, lane b'). In contrast, lanes a, a', d, d', e, and e' contained monoclonal antibodies that recognized epitopes located on the major outer membrane protein, as determined by immunoblotting (data not shown). The immunoreactivity of these epitopes was unaffected by NaIO4 treatment.

The L21-6 monoclonal antibody was an IgG3 molecule, as determined by double-immunodiffusion analyses in which subclass monospecific goat anti-mouse IgG antiserum was used (data not shown). The genus-specific epitope recognized by monoclonal antibody L21-6 and a type-specific epitope located on the LPS molecule. All but one of these monoclonal antibodies appeared to be immunodominant determinants when Formalin-fixed EBs were used as the immunogen. Using the procedures described in this study, we identified 16 hybridomas secreting chlamydial antibodies obtained from three different BALB/c spleen-NS1 cell fusions. Six (37%) of these hybridomas recognized the periodate-sensitive, genus-specific epitope, and 8 (48%) reacted with an L2 serovarspecific epitope located on the major outer membrane protein of the organism. All but one of these monoclonal antibodies were of the IgG3 subclass.

Isolation of chlamydial LPSs. The NaIO4 sensitivity and the genus prevalence of the epitope recognized by monoclonal antibody L21-6 indicated that the antigen may be the chlamydial glycolipid antigen. Since Nurminen et al. (24) have recently shown that the chlamydial glycolipid antigen is an LPS molecule, we attempted to isolate the glycolipid antigen with LPS extraction procedures. Initially, L2 EBs (65 mg [dry weight]) were extracted by the method described by Galanos, but we were unable to precipitate LPS from the phenol phase. The PCP residue was reextracted by the hot phenol-water method of Westphal and Jann (33) and yielded a small amount of LPS (0.3%, on a weight basis). Extraction of EBs directly by the hot phenol-water method resulted in almost no aqueous phase and a large interface. This observation, coupled with the minimal success obtained with the Galanos procedure, led us...
TABLE 1. Characterization and comparison of Chlamydia and Salmonella RE LPSs

<table>
<thead>
<tr>
<th>LPS</th>
<th>Starting material</th>
<th>Extraction method</th>
<th>Yield (%)</th>
<th>Protein (%)</th>
<th>KDO (%)</th>
<th>LAL test result</th>
<th>Total hexoses and pentoses (%)</th>
<th>Reactivity with:</th>
</tr>
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<tbody>
<tr>
<td>C. trachomatis L2</td>
<td>Reduced and alkylated EBs</td>
<td>P-W</td>
<td>1.9</td>
<td>&lt;1.0</td>
<td>8.8</td>
<td>+</td>
<td>10.5</td>
<td>+ + +</td>
</tr>
<tr>
<td>S. typhimurium LT2, Re mutant</td>
<td>SA1 no. 377 Re whole cells</td>
<td>PCP</td>
<td>3.0</td>
<td>2-3.0</td>
<td>19.3</td>
<td>+</td>
<td>5.0</td>
<td>+ + +</td>
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a P-W, Phenol-water (see text).
b Percent protein was determined on a weight basis by the method of Lowry et al. (21).
c Percent KDO was determined on a weight basis (11, 25).
d LAL, Limulus amoebocyte lysate (Difco). Both LPSs were positive at >0.1 ng/ml.
e Numbers represent the means of values obtained in two separate experiments and determined by the phenol-sulfuric acid method of Dubois et al. (14). This procedure is suitable for the estimation of total carbohydrates in bacterial cells that contain ≥10% hexose polymers. The pentoses in nucleic acid interfere when hexose content is low. The difference between total hexoses and pentoses of S. typhimurium Re mutant and C. trachomatis cannot, therefore, be regarded as significant.
f Monoclonal antibody L21-6 reacted with the genus-specific LPS epitope.

FIG. 2. Immunoblot of phenol-water-isolated chlamydial LPS reacted with monoclonal antibody L21-6. Lanes a through e, silver-stained polyacrylamide gel; lanes a' through e', accompanying immunoblot. Lanes a and a', L2 serovar whole-cell lysate (20 μg of protein electrophoresed); lanes b and b', the same L2 serovar whole-cell lysate treated with proteinase K; lanes c and c', 5 μg of phenol-water-extracted LPS of L2 EBs; lanes d and d', L cell residue after harvesting L2 organisms; lanes e and e', uninfected L cells. Note that monoclonal antibody bounds to the chlamydial LPS isolated by the phenol-water procedure. Also note that the immunoreactivity and migration of the LPS was unaffected by proteinase K treatment; however, the non-immunoreactive polypeptides were completely digested by the protease (cf. lanes a and b). LPS was detectable in the L cell debris recovered after harvesting chlamydiae (lanes d and d') but not in uninfected L cells (lanes e and e'). Notice that a high-molecular-weight component that reacted with monoclonal antibody L21-6 was present in infected L cell debris (lanes d and d'). This material was not detected in whole-cell lysates of L2 EBs, purified chlamydial LPS, or uninfected L cell controls.

to speculate that the chlamydiae behaved atypically in regard to the partitioning of outer membrane macromolecules by both of these LPS extraction methods. It is known that the major outer membrane protein of the chlamydial EB is extensively cross-linked by disulfide bonds (23; T. Hackstadt, unpublished observation). We felt that these covalent interactions may have interfered with the LPS extraction procedures and were partly responsible for our inability to quantitatively isolate chlamydial LPS. Therefore, we first treated purified EBs with dithiothreitol, blocked reduced sulfhydryls with iodoacetamide, and then subjected these preparations to both LPS extraction procedures. A total of 0.625 mg of LPS (1.9% yield) was recovered from 32.6 mg of reduced and alkylated EBs by the phenol-water procedure (Table 1). However, even using reduced and alkylated EBs, we were unable to extract LPS by the Galanos procedure. Preliminary characterization of chlamydial LPS is summarized in Table 1. Parallel analyses of S. typhimurium Re LPS were done. Both LPSs had similar activities in a Limulus amoebocyte lysate assay. The KDO values were dissimilar. Although reproducible analytical figures were obtained, they did not indicate the true KDO content of the samples. Not only is KDO labile, it is known that the molar absorption of KDO varies considerably with the presence of substituents (11). The low values obtained for total carbohydrates in chlamydial and Re LPSs by the phenol-sulfuric acid method of Dubois et al. (14) were at or below the limits of reliability for the assay. The test results were indicative of low hexose contents in both LPSs.

Location of the genus-specific epitope on chlamydial LPS. Immunoblotting analysis was used to determine whether the genus-specific epitope recognized by monoclonal antibody L21-6 was located on chlamydial LPS (Fig. 2). The following preparations were electrophoresed and immunoblotted: (i) L2 EB whole-cell lysate without (lanes a and a') and with (lanes b and b') proteinase K treatment; and (ii) phenol-water-isolated chlamydial LPS (lanes c and c'), phenol-water extract of the cellular debris recovered after harvesting chlamydiae from infected L cells (lanes d and d'), and an uninfected L cell control (lanes e and e'). Several points can be made from the data presented in Fig. 2. First, the silver-stained polyacrylamide gel shows that the phenol-water-
isolated chlamydial LPS (lane c) is homogeneous and migrates as a low-molecular-weight molecule ($M_r \leq 10,000$). As shown in the accompanying immunoblot (lane c'), monoclonal L2-6 intensely binds to isolated chlamydial LPS and to material in the whole-cell and digested lysates which had similar electrophoretic mobility. These findings show that the genus-specific epitope recognized by monoclonal antibody L21-6 is located on chlamydial LPS. Hitchcock and Brown (18) have previously shown that sodium dodecyl sulfate-polyacrylamide gel electrophoresis of protein K-digested whole-cell bacterial lysate can be used for comparative analysis of LPS. This technique allows analysis of LPS to be done on extremely small quantities of complex mixtures of macromolecules, a particularly attractive feature for the study of chlamydial LPS because of the extreme difficulty in obtaining large quantities of this organism. The results of this technique for the study of chlamydial LPS are shown in Fig. 2 (lanes a and b). Lane a in Fig. 2 shows the complex silver-staining profile obtained after electrophoresis of ca. 5 \times 10^7 L2 EBs solubilized in Laemmli sample buffer. Lane b contained an identical preparation, except the lysate was incubated with proteinase K before electrophoresis. The fast-migrating, intensely silver-stained band near the bottom of the gel (arrow) is chlamydial LPS, as shown by its comigration with isolated LPS (lane c) and immunoreactivity with monoclonal antibody L21-6 (lanes a' and b'). Lanes d and d' contained electrophoresed L-929 cell debris recovered from infected L cells after harvesting chlamydiae, and lanes e and e' were uninfected L cell controls. A substantial amount of LPS was present in L cell debris from which chlamydiae had been harvested (lane d). Additionally, in the L cell residue, there was silver-staining material which failed to enter the separating gel. This high-molecular-weight material contained chlamydial LPS, since it bound monoclonal antibody L21-6 (lane d'). This material was not observed in the EB whole-cell lysate, isolated chlamydial LPS, or uninfected L cells. The nature of this immunoreactive, high-molecular-weight component is unknown. It is curious, however, that the material is associated only with the chlamydial infected L cell debris, suggesting that it may be chlamydial LPS that is tightly associated with host macromolecules.

Specificity of monoclonal antibody L21-6 for chlamydial LPS. The findings presented above show that monoclonal antibody L21-6 recognized an epitope located on chlamydial LPS. Although our preliminary data indicate that this epitope was common to the genus (Fig. 1), we further examined the reactivity of monoclonal antibody L21-6 against each of the 15 C. trachomatis serovars and seven different C. psittaci strains by microimmunofluorescence (Table 2). Antibody L21-6 reacted at an identical endpoint titer of 1:320 with the acetone-fixed EBs of all chlamydiae tested. For purposes of comparison, the specificity of monoclonal antibodies L21-45 and L21-10 that recognize type-specific and subspecies-specific epitopes located on the major outer membrane protein, respectively, are also shown in Table 2. Monoclonal antibody H5332 was included as a negative control. These serological data show that antigenic determinant seen by antibody L21-6 was common to chlamydial isolates of both species.

<table>
<thead>
<tr>
<th>TABLE 2. Specificity of monoclonal antibody L21-6 by microimmunofluorescence</th>
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<tr>
<td><strong>Chlamydia serovar or strain</strong></td>
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<tr>
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<tr>
<td><strong>C. trachomatis serovar</strong></td>
</tr>
<tr>
<td>A</td>
</tr>
<tr>
<td>B</td>
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<tr>
<td>Ba</td>
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<td>L1</td>
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<td>L2</td>
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<tr>
<td>L3</td>
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<tr>
<td>Mouse pneumonia</td>
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<td><strong>C. psittaci</strong></td>
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<td>Bovine abortion 8390</td>
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<tr>
<td>Feline pneumonia 562</td>
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<tr>
<td>Guinea pig inclusion conjunctivitis</td>
</tr>
<tr>
<td>Meningopneumonitis Cal-10</td>
</tr>
<tr>
<td>6BC</td>
</tr>
<tr>
<td>Parrot/Meyer</td>
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<td>Parakeet/Arizona</td>
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*Reciprocal of highest dilution resulting in positive microimmunofluorescence (micro-IF). L21-6, Chlamydial LPS; L21-45, major outer membrane protein L2 serovar-specific epitope; L21-10, major outer membrane protein C. trachomatis subspecies epitope; H5332, negative control monoclonal antibody against an outer membrane protein of the Lyme disease spirochete. –, Negative at a dilution of 1:10.
The data presented above demonstrate that the epitope under study was shared by all members of the genus *Chlamydia*. We next attempted to determine whether this epitope was chlamydia specific or a determinant present on the LPS of other gram-negative organisms. We tested the immunoreactivity of antibody L2I-6 against six chlamydial isolates, *N. gonorrhoeae* JS1, *S. typhimurium*, and *Escherichia coli* by immunoblotting. Both whole-cell lysate preparations and isolated LPS were analyzed for each organism.

Additionally, several *S. typhimurium* strains which represented different LPS chemotypes were tested. These were *S. typhimurium* smooth, Ra, Rd2, and Re LPSs. Isolated smooth and rough LPS of *E. coli* O111:B4 and mutant J5 were also tested. The silver-stained profiles and their accompanying immunoblot against monoclonal antibody L2I-6 are shown in Fig. 3. The LPS of each chlamydial strain reacted strongly with this antibody (lanes a' through f'); however, LPSs from *N. gonorrhoeae*, *S. typhimurium*, and *E. coli* were not reactive (lanes g' through q'). In addition, isolated LPSs of *Coxiella burnetii* (phase I or II) and *Rickettsia rickettsii* did not react with antibody L2I-6 by immunoblotting (Ted Hackstadt, personal communication, and unpublished observations by H.C.). These immunological findings show that, at least for the limited number of extracellular and obligate intracellular bacteria examined, the genus-specific epitope recognized by monoclonal antibody L2I-6 is unique to chlamydiae and is not shared by the LPS of other gram-negative bacteria.

**Antigenic properties of chlamydial LPS.** To further explore the antigenic properties of chlamydial LPS, we tested the reactivity of polyclonal rabbit antisera raised against isolated *S. typhimurium* Re LPS and the lipid A portion of the macromolecule against six chlamydial strains, the isolated LPSs of *N. gonorrhoeae*, and the smooth, Ra, and Re LPSs of *S. typhimurium*. The silver-stained profiles of each of these preparations are described above (Fig. 3). The immunoblotting results are shown in Fig. 4. The location of chlamydial LPS is identified in the figure by arrows. Rabbit antiserum raised against Formalin-killed L2 serovar EBs reacted with LPS of each of the chlamydial strains tested. It failed to react with the LPS of *N. gonorrhoeae*, or the smooth and Ra LPSs of *S. typhimurium*. There was, however, a very weak but discernible reaction with the Re LPS of *S. typhimurium* (not apparent in the figure). The reactivity of rabbit antiserum specific for the Re LPS of *S. typhimurium* against the same preparations is also shown in Fig. 4. Each of the chlamydial LPSs reacted strongly with this antiserum, as did isolated Re LPS of *S. typhimurium*. Interestingly, no reaction was observed with either the smooth or Ra LPS. This antisera does not react with chemotypes Ra through Rd2 (unpublished data). In contrast to this apparent specificity observed with anti-Re LPS serum, anti-*S. typhimurium* lipid A serum reacted with chlamydial LPS, *N. gonorrhoeae* LPS, and the smooth, Ra, and Re LPSs of *S. typhimurium*. These results demonstrate that the chlamydial LPS shares antigenic determinants with the LPS of *S. typhimurium* and that the structure of the chlamydial LPS may be very similar to the *Salmonella* Re chemotype LPS (containing similar lipid A and KDO constituents).

**DISCUSSION**

We describe in this work a monoclonal antibody, L2I-6, that recognizes a chlamydial genus-specific epitope. We show that the epitope is shared by all members of the genus *Chlamydia* and that the epitope is located on the LPS of these obligate intracellular procaryotes.

Nurminen et al. (24) recently reported that the chlamydial glycolipid group antigen is LPS and that the isolated chlamydial LPS is antigenically similar to the Re LPS of *S. typhimurium*. These conclusions are based on the observation that, by enzyme immunoassay, rabbit antiserum raised against Re mutants of *S. typhimurium* and *S. minnesota*, but not hyperimmune antiserum raised against isogenic Rd2 or Ra LPS mutants, reacted with isolated chlamydial LPS. Our data (Fig. 4) support the findings of these investigators. In
addition to their observations, however, we showed that the chlamydial LPSs possessed at least three distinct antigen domains, two of which were shared by other gram-negative organisms and one (the chlamydial genus-specific epitope recognized by monoclonal antibody L21-6) that was common only to members of the genus Chlamydia. The shared determinants appeared to be restricted to the KDO and lipid A part of the LPS molecule (Fig. 4). Aside from the periodate sensitivity suggestive of carbohydrate, the chemical nature of the chlamydial genus-specific LPS epitope is unknown; however, studies are under way in this laboratory to chemically define this antigen.

Stephens et al. (29) have recently produced monoclonal antibodies against chlamydial antigens. These investigators described a monoclonal antibody, 2-H11, which reacts with a genus-specific pattern by microimmunofluorescence. This antibody apparently reacts with an epitope on the chlamydial LPS, since, by immunoblotting, the immunoreactive component migrates as a single species with a subunit molecular weight of >10,000. The determinant described by these authors is also sensitive to periodate treatment. Since these investigators did not analyze the reactivity of their 2-H11 monoclonal antibody against other gram-negative organisms, it is unclear as to whether the epitope is the same chlamydial genus-specific LPS determinant described in our studies.

The antigenic relatedness observed between chlamydial LPS and the LPS of S. typhimurium is particularly interesting and deserves some comment. Rabbit antiserum against S. typhimurium Re LPS bound chlamydial LPS and the homologous Re LPS of Salmonella spp. (Fig. 4). However, this antiserum failed to bind to either the Ra or smooth Salmonella LPS or chemotypes with additional core sugars Ra through Rd2 (unpublished data). These findings are somewhat perplexing but are in agreement with the suggestion of Lüderitz et al. (22) that the presence of core oligosaccharides and O-polysaccharides adjacent to the KDO di- or trisaccharide “shield” the KDO components from reacting with antibody. It is also possible that the chlamydial LPS may have additional sugars bound to KDO; for unknown reasons, however, they do not sterically inhibit the binding of Re antibodies to the KDO. An alternative explanation is that the epitope recognized by the Re antibodies is a substituent that is not present or accessible on the KDOs of other salmonellae chemotypes. The fact that lipid A antibodies bind only a relatively small area of the LPS and not the entire ladder of the smooth LPS may also be the result of shielding effects of core and O-saccharides.

Our findings show that quantitative yields of chlamydial LPS were achieved only when EBs were first reduced and alkylated and then extracted by the hot phenol-water procedure. These observations are possibly peculiar to chlamydiae and may reflect the rather unique outer membrane and cell wall structure of these organisms. For example, we have previously shown that C. trachomatis lacks detectable muramic acid, indicating the absence of a typical peptidoglycan structure (1). The ability of Chlamydia organisms to maintain a rigid shape in the absence of peptidoglycan is believed to occur via the major outer membrane protein. The chlamydial major outer membrane protein constitutes 65% or more of the total outer membrane protein (8) and is known to be extensively cross-linked in the EB form of the parasite by disulfide bonds (23; unpublished observations). Our findings indicate that these covalent bonds which occur between this structural protein prevent its complete partitioning into the hot phenol phase, resulting in atypical extraction results (i.e., large interface phase). Examination of interface material revealed primarily large amounts of major outer membrane protein and LPS (data not shown). Reduction of EBs with dithiothreitol significantly increased the yield of chlamydial LPS (Table 1). What the specific relationship is between the reduced major outer membrane protein and the

FIG. 4. Immunoblot of LPS of chlamydiae and other gram-negative organisms with rabbit polyclonal anti-chlamydial EB: anti-S. typhimurium Re LPS and anti-lipid A IgG (80 μg of IgG per ml was used for preparation). The immunoreactivity of rabbit anti-L2 serovar EB IgG is shown in the left-hand plate. Antibodies reactive with the LPS of each chlamydial strain are evident (arrow). A very weak but discernable reaction also is present with the S. typhimurium chemotype Re LPS. No reaction was observed with the S. typhimurium chemotype smooth or Ra LPSs or the isolated LPSs of N. gonorrhoeae JS1. In the middle plate, the immunoreactivity of rabbit anti-S. typhimurium Re LPS is shown. Note that the anti-Re LPS antibodies intensely bound each chlamydial LPS and the homologous S. typhimurium Re LPS but not the smooth, Ra, or gonococcal LPS. The immunoreactivity of the anti-Re LPS was very similar to that observed for the anti-EB IgG shown in the left-hand plate. In the right-hand plate, the immunoreactivity of rabbit anti-S. typhimurium lipid A is shown. Note that the anti-lipid A IgG reacted with the LPS of Chlamydia, N. gonorrhoeae, and each of the S. typhimurium chemotype LPSs. The nature of the high-molecular-weight immunoreactive components present in the immunoblots probed with anti-Re or anti-lipid A IgG are unknown. No reaction was observed with the chlamydial LPS when blots were incubated with a 1:100 dilution of pooled normal rabbit serum (data not shown). Approximately 20 μg of protein was electrophoresed for each whole-cell lysate and 5 μg (dry weight) of isolated LPSs.
extractability of LPS is presently unknown. It should be noted, however, that even after reduction of EBs with dithiothreitol, we were unsuccessful in isolating LPS by the PCP procedure of Galanos. These findings were somewhat disturbing, since Nurminen et al. (24) used the PCP procedure for isolating chlamydial LPS from the same serovar used in this study. It should be pointed out, however, that the above investigators did not report on the yields of chlamydial LPS they obtained by the PCP method. It is possible, therefore, since quantitative data were not presented, that only minimal yields of chlamydiae LPS were obtained. We do not understand why the PCP method was unsuccessful in our hands for isolating chlamydial LPS. In fact, considering the apparent structural similarity between chlamydial LPS and the Re LPS of Salmonella spp., the PCP method should have been superior to the hot phenol-water extraction. A possible explanation for our results is that excess salt ions, which have been previously reported to interfere with LPS extraction by the PCP method (16), were present in our EB preparations. Nevertheless, we are confident that respectable yields of chlamydial LPS (2%) can be isolated by the procedures described here. These quantities of LPS were sufficient for detailed chemical analyses of this molecule. Additionally, since reasonable amounts of chlamydial LPS are now obtainable, the biological properties of this molecule can be studied. The chlamydial LPS may be an interesting functional component with respect to chlamydiae-host cell interactions. For example, all chlamydiae share a common surface component(s) which functions in inducing their uptake by endocytosis (7) and in preventing phagolysosome formation after uptake of the chlamydia-laden endosome (15). This common denominator of virulence is shared by all chlamydiae, even though there is a tremendous diversity in both the antigenic structure (9) and genetic composition (19, 27) between the two Chlamydia species. Although purely speculative at this point, it does not seem unreasonable to suggest that, given the diversity of pharmacological activities of different LPSs, the chlamydial LPS, which is located in the outer membrane and is a conserved structure common to all chlamydiae, may be an important virulence determinant of these obligate intracellular parasites.

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LITERATURE CITED


