Accumulation of Chlamydial Lipopolysaccharide Antigen in the Plasma Membranes of Infected Cells

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The presence of a chlamydia-specific antigen associated with the plasma membrane of infected cell lines was demonstrated by indirect immunofluorescence staining with a monoclonal antibody, designated 47A2, specific for the chlamydial genus-specific lipopolysaccharide (LPS) antigen. Staining of HeLa, L-929, and McCoy cells infected with the L2 or F serovar of Chlamydia trachomatis was observed either without fixation or following aldehyde fixation and brief drying. The 47A2-reactive antigen appeared to be present on the plasma membrane, on bleb-like structures on the host cell surface, and on proximal processes of neighboring uninfected cells. Antibodies to chlamydial protein antigens such as the major outer membrane protein produced no surface staining under similar conditions. Membrane vesicles elaborated from infected cells were enriched for the 47A2-reactive antigen. Superinfection of chlamydia-infected cells with vesicular stomatitis virus, an enveloped virus which buds from the plasma membrane, allowed purification of progeny virions that were enriched with chlamydial LPS. These results are consistent with the presence of chlamydial LPS in the plasma membranes of infected host cells.

The life cycle of Chlamydia trachomatis requires a variety of interactions between the bacterium and membranes of the host cell; both the plasma membrane and internal membrane structures of the host cell are involved in the chlamydial growth cycle. This obligate intracellular pathogen must interact with the cell surface to gain access to the host cell (1, 8, 13). Once the pathogen is inside the cell and enclosed in a membrane-bound vesicle, or phagosome, the fusion of phagosomal and lysosomal membranes is inhibited (12, 35), allowing the bacteria to remain in a relatively isolated intracellular environment. In addition, while residing within the host cell, chlamydiae seem to be relatively resistant to host immune cytolysis (16, 24, 25) which, to be effective during the intracellular tenure of the organism, must surmount the barrier of the host cell plasma membrane (33, 35). The immune responses stimulated during chlamydial infection include those that may be targeted against chlamydia-specific structures on the infected host cell surface.

Our initial hypothesis was that since chlamydiae behave as intracellular pathogens, they may deposit on the surface of the infected cell an antigen that is chlamydia specified in a manner analogous to that observed for viruses (11). Initial attempts to test this hypothesis utilized surface iodination of infected cells to identify on sodium dodecyl sulfate gels novel protein bands that were not evident in samples from control cells; these attempts were unsuccessful and revealed no difference between infected and uninfected cells. In addition, no chlamydia-specific antigen was recovered following immunoprecipitation of 125I-surface-labeled infected cells with polyclonal antisera.

Using polyclonal antichlamydia antibodies, Richmond and Stirling (26) identified a chlamydia-derived antigen on the surface of infected cells by indirect immunofluorescence staining of Formalin-fixed and dried cells. To identify the specific antigen(s) responsible for this reactivity, we used a similar approach with a panel of monoclonal antibodies. The reactivity of one antibody, 47A2, with the surface of fixed and unfixed infected cells indicated that the chlamydial lipopolysaccharide (LPS) becomes associated with the cell plasma membrane during chlamydial infection. This association was confirmed by demonstrating an enrichment for chlamydial LPS in vesicles shed from infected cells and in virions of vesicular stomatitis virus (VSV) purified following superinfection of chlamydia-infected cells.

A preliminary report of some of these experiments was presented previously [34].

MATERIALS AND METHODS

Chlamydial strains and growth conditions. C. trachomatis L2/434/Bu and F/UW6/Cx were used in these studies; chlamydia strains were supplied from the reference collection of the Division of Infectious Diseases, Indiana University School of Medicine. Stocks of chlamydiae were harvested from McCoy, HeLa-229, or L-929 cell monolayers in 150-cm² flasks as described previously (20).

Monoclonal antibodies. The production of murine monoclonal antibodies 1A5 and F221/6C2/C2 has been described previously (3); these antibodies are specific for the major outer membrane proteins of serovars L2 and F, respectively. Antibody 47A2 was recovered following fusion of splenocytes from a BALB/c mouse that had been immunized with elementary bodies of strain L2/434/Bu to the SP2/0-Ag14 cell line as described previously (3). All antibodies were used as unfractionated ascites fluids.

Indirect immunofluorescence staining. A modification of the method of Richmond and Stirling (26) was used to examine chlamydia-infected cultures for the distribution of chlamydial antigens. At different times postinoculation, chlamydia-infected and uninfected control monolayers on cover slips were rinsed with phosphate-buffered saline (PBS) and fixed in either 4% formaldehyde or 4% glutaraldehyde in PBS for 20 min (26). Following brief air drying (30 min) at room temperature, monolayers were treated with dilutions of monoclonal antibodies in PBS for 1 h at room temperature. After three PBS washes, cells were treated with

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fluorescein isothiocyanate (FITC)–rabbit anti-mouse immunoglobulin G (IgG) for 1 h in the dark and washed three times with PBS. Alternatively, cell monolayers were stained in the absence of any fixation or drying. Photomicrographs were taken by using a Zeiss epifluorescence microscope with a 35-mm photomicrographic camera.

**Dot blot immunoassay.** The relative amounts of chlamydial antigens in various samples were measured by a dot blot immunoassay. Samples were solubilized in 0.2% sodium dodecyl sulfate, and appropriate dilutions were applied to nitrocellulose paper in 100-μl aliquots with dot blot manifold (Bio-Rad Laboratories, Richmond, Calif.). After antigen application, the nitrocellulose paper was blocked by treatment for 1 h with 1% bovine serum albumin in PBS. Following incubation for 2 h in a saturating dilution of anti-chlamydia monoclonal antibody, the paper was washed, incubated for 1 h with rabbit anti-mouse immunoglobulin, and washed, and immune complexes were detected with 125I-protein A by autoradiography and gamma scintillation counting.

**Vesicle induction and purification.** To induce vesiculation, we washed L4/343/Bu-infected and control L-929 cultures with PBS containing 0.75 mM CaCl2 and 0.5 mM MgCl2 and treated with 25 mM Formalin and 2 mM dithiothreitol (DTT) in PBS with CaCl2 and MgCl2, for 2 h at 37°C (28). Alternatively, infected and control HeLa cells were treated with cytochalasin D (2.5 μg/ml) and vinblastine (10 μg/ml) in Hanks balanced salt solution for 1 h at 37°C. Culture supernatants containing released vesicles were passed over a glass wool column to remove any detached cells; vesicles were washed three times by centrifugation at 3,000 × g for 30 min. Samples of vesicle preparations were plated on cover slips treated with poly-L-lysine (1 mg/ml in PBS), fixed with 4% formaldehyde in PBS, and stained by indirect immunofluorescence. Additional samples were solubilized in 0.2% sodium dodecyl sulfate, and their antigenic compositions were evaluated by the dot blot immunoassay.

**VSV superinfection.** In these experiments, chlamydia-infected cells were superinfected with VSV; progeny virions were purified (see below), and the relative amounts of LPS and major outer membrane protein (MOMP) antigens present in the viral envelopes were determined serologically. Stocks of VSV (Indiana serotype) grown in L-929 cells were used throughout these studies; viral titers were determined by a plaque assay on L-929 cells (31).

Monolayers of L-929 cells were inoculated with either the L4/343/Bu or the F/UW6/Cx strain of chlamydiae; following incubation for 20 h (L4/343/Bu) or 40 h (F/UW6/Cx), the cultures were superinfected with VSV at a multiplicity of infection of 5. Eight hours following viral inoculation, culture supernatants were collected and the progeny virions were purified by the methods of McSharry and Wagner (17). Culture fluids were centrifuged at 1,000 × g for 15 min. The supernatants were transferred into polyallomer tubes over a 2-ml cushion of 50% glycerol in Hanks balanced salt solution; following centrifugation at 104,000 × g (SW27 rotor; Beckman Instruments, Inc., Fullerton, Calif.) for 90 min at 4°C, the pellet was suspended in 20 mM Tris hydrochloride–0.15 M NaCl (pH 7.2) and layered over a continuous 10 to 40% sucrose gradient in the same buffer. The gradients were centrifuged at 47,000 × g (SW27 rotor; Beckman) for 90 min at 4°C. A band of VSV which formed in the 30 to 40% sucrose area was collected, and the virus was concentrated by centrifugation at 83,000 × g.

**Other analytical methods.** Immunoelectrophoretic transfer analysis was performed as previously described (2). Ketodeoxyoctonate was determined by the thiobarbituric acid method (32), while the gelation of Limulus amebocyte lysate was performed according to the instructions of the manufacturer (Associates of Cape Cod, Woods Hole, Mass.). Statistical analysis was done with Student’s t test.

**RESULTS**

**Antigenic specificity of monoclonal antibody 47A2.** Antibody 47A2 reacted with an antigen present in elementary and reticulate bodies of all serovars of *C. trachomatis* and with a strain of *C. psittaci*. Immunoelectrophoretic transfer analysis indicated that the 47A2-reactive antigen migrated with the tracking dye on a conventional sodium dodecyl sulfate–10% polyacrylamide gel but could be resolved on a 15% gel. The antigen was insensitive to the action of proteases but was sensitive to periodate oxidation. Partially purified antigen induced the gelation of *Limulus* amebocyte lysate at 10-ng/ml quantities and contained 3.1% ketodeoxyoctonate. Antibody 47A2 reacted with LPS purified from *C. psittaci* (provided by P. B. Wyrick, University of North Carolina School of Medicine, Chapel Hill) but failed to react with gram-negative bacteria such as *Escherichia coli*, *Proteus vulgaris*, *Neisseria gonorrhoeae*, and *Acinetobacter calcoaceticus*. Finally, immunoelectrophoretic transfer analysis showed that 47A2 reacted with a low-molecular-weight, periodate-sensitive, protease-resistant antigen expressed by *E. coli* JMI09 harboring the recombinant plasmid pFEN207 (19), which contains a fragment of chlamydial chromosome (the generous gift of F. E. Nano, Rocky Mountain Laboratories, Hamilton, Mont.); this recombinant strain expresses, in addition to the parental complete *E. coli* LPS, a second, rough-type LPS which bears a chlamydia-specified genus-specific epitope (4, 19). Antibody 47A2 did not react with *E. coli* JMI09 carrying the vector plasmid pUC8 which lacks the chlamydial DNA insert. These data indicate that 47A2 recognizes a genus-specific epitope which is part of the chlamydial LPS.

**Immunofluorescence staining of cells infected with *C. trachomatis*.** Indirect immunofluorescence staining of cells infected with *C. trachomatis* indicated that monoclonal antibody 47A2 produced staining of the surface of chlamydia-infected cells that had been Formalin fixed and air dried. The distribution of 47A2-reactive antigen as detected with FITC–anti-IgG coincided with the plasma membranes of infected cells (Fig. 1). In many cases, the staining appeared on bleblike structures on the surface of infected cells and on the proximal processes of neighboring cells. This pattern was observed for all chlamydial serovars examined and was correlated with the degree of chlamydial infection; uninfected cells, with no inclusions discernible by phase microscopy (Fig. 1), and uninfected control cultures (data not shown) showed no staining following identical treatment. Furthermore, the expression of the 47A2-reactive antigen was independent of host cell type, with equivalent staining patterns being observed for infected HeLa, McCoy, and L-929 cells. Monoclonal antibodies directed against another chlamydial antigen, the MOMP, produced no surface staining under these conditions (data not shown).

In contrast to the distribution of antigen observed following Formalin fixation, methanol-fixed monolayers demonstrated staining patterns restricted to the chlamydial inclusions, indicating that the LPS antigen recognized by antibody 47A2 was present in the chlamydial inclusions. Furthermore, the lack of apparent surface staining of methanol-fixed cells suggested that the antigen was extracted.
Vesiculation. Treatment of L2/434/Bu-infected L-929 cells with Formalin and DTT induced a rapid shedding of vesicles in the size range of 10 to 35 μm. Indirect immunofluorescence staining of aldehyde-fixed vesicles with antibody 47A2 produced relatively uniform surface staining at intensities comparable to those obtained for intact infected cells (Fig. 3A), but there was little or no staining with anti-MOMP antibodies (Fig. 3B). Vesicle shedding was induced at various times following chlamydial infection; the relative ratios of LPS and MOMP were determined by the dot blot immunonassay with specific monoclonal antibodies and were compared with the ratios obtained for unfractionated lysates of infected cells obtained at the same times. The relative LPS content of vesicles was lower than that of intact infected cells during the early stages of infection but increased sharply at 25 h (Fig. 4). It may be noteworthy that this increase occurred coincident with the commencement of condensation of reticulate bodies to elementary bodies (27). Vesicles induced by treatment with cytochalasin D and vinblastine yielded similar results, although vesicle size was more uniformly distributed around 10 μm. Table 1 summarizes the LPS/MOMP ratios obtained for vesicles induced in different ways from L2/434/Bu-infected HeLa cells as compared with infected HeLa cell lysates and purified elementary bodies and reticulate bodies. When compared with infected cell lysates, all vesicles demonstrated enrichment with LPS.

Superinfection with VSV. To confirm that chlamydial LPS was being incorporated in the plasma membrane of infected cells, the cells were superinfected with VSV. Since the envelopes of the progeny virions are derived from the host cell plasma membrane (17, 36), those particles arising from chlamydia-infected cells should be enriched with chlamydial LPS.

L-929 cells were infected with C. trachomatis L2/434/Bu for 20 h and superinfected with VSV. Preliminary experiments indicated that, by 8 h following viral inoculation, cell rupture was slight and progeny VSV titers in the culture medium were high (around 3.7 × 10⁸ PFU/ml). Progeny virions released into the culture medium were purified on sucrose gradients. In the case of chlamydia-infected cells, a pellet of material composed of chlamydial particles and cellular fragments formed at the bottom of the gradient tubes. The pellet, in addition to the VSV band, was analyzed for the amounts of LPS and MOMP with monoclonal antibodies 47A2 and 1A5, respectively. The LPS/MOMP ratio in purified virions was more than twice that in the gradient pellet and purified elementary bodies (Table 2).

The presence of MOMP reactivity in the purified virion preparations suggested contamination with chlamydial organisms. Repurification of the virions on a second sucrose gradient resulted in a recovery of 33% of the LPS reactivity with no detectable MOMP reactivity (Table 2). To determine whether the chlamydial antigens were adventitiously associated with the VSV virions, control virions were admixed with 90% of the pellet acquired from the chlamydia-infected cultures. Following repurification, little or no chlamydial antigens were detectable in the virion preparations. This finding also indicates that the method of virion purification effectively eliminated contamination with intact chlamydiae.

Similar results were obtained with C. trachomatis F/UW6/Cx. In this case, recovered virions were enriched with 47A2-reactive antigen by a factor of five as compared with unfractionated lysates of infected cells and by a factor of 10 as compared with isolated elementary bodies (Table 2). The greater enrichment probably resulted from the longer time of
chlamydial infection prior to VSV inoculation: 40 h for F/UW6/Cx versus 20 h for L2/434/Bu.

DISCUSSION

Chlamydiae are obligate intracellular bacteria which appear to possess a cell envelope similar in some respects to that of gram-negative bacteria. Although apparently lacking a murein sacculus (14), chlamydiae have both inner and outer membranes (29). Among the components of this envelope are the MOMP, which may substitute structurally for the peptidoglycan (15, 21), and LPS. Chlamydial LPS is structurally reminiscent of the deep rough LPS of Re strains of Salmonella spp., including a trisaccharide of ketodeoxyoctonate but lacking additional core polysaccharides and O side chains (5, 22, 23). The novel 2→8 linkage of the second and third ketodeoxyoctonate moieties is the major contributing structure to the antigenic epitope recognized by various “group-specific” serological reagents (4, 6, 7, 9, 10, 30), including monoclonal antibody 47A2 used in these studies.

The goal of this study was to identify a chlamydial antigen in the plasma membrane of infected eucaryotic host cells. Upon screening a panel of monoclonal antibodies by the immunofluorescence staining technique of Richmond and Stirling (26), one antibody was identified, designated 47A2, which stained the surface of chlamydia-infected cells. Determination of the antigenic specificity of 47A2 indicated that it reacted with chlamydial LPS. The surface staining was chlamydia dependent; i.e., staining was observed on infected McCoy, HeLa, and L-929 cells, while uninfected cultures were devoid of staining. Staining was first detect-
TABLE 1. Ratios of LPS/MOMP reactivities in vesicles shed from L.\textsubscript{/434}/Bu-infected cells, infected cell lysates, and isolated chlamydial membranes which, in turn, may influence the degree of epitopic exposure of incorporated LPS.

To confirm the presence of the 47A2-reactive antigen in the plasma membrane of infected cells, we wished to acquire fractions of the plasma membrane without markedly disturbing the chlamydial inclusions. This goal was accomplished by two methods. In the first case, extrusion of membrane vesicles was induced by treatment of infected cells with agents which perturb the cell surface and cytoskeleton. In the second case, the budding of progeny virions following VSV superinfection provided a preparation that was rich in plasma membrane and that should have been relatively deficient in both intracellular membrane material and chlamydial organisms. Studies of the lipid composition of purified VSV have indicated that viruses propagated in L-929 cells have a lipid composition very similar to that of the L-929 cell plasma membrane but distinct from that of the L-929 intracellular membrane (17). Therefore, it was assumed that the envelope of VSV originating from chlamydia-infected cells was representative of the infected cellular plasma membrane. Serological analyses of these preparations confirmed that they were enriched with chlamydial LPS, although some contamination with chlamydial organisms was evident as reactivity with anti-MOMP antibodies. Nonetheless, the LPS/MOMP ratio was greater in these preparations than in solubilized elementary bodies, reticulate bodies, or lysates of infected cells.

The LPS destined to appear in the plasma membrane may originate during the morphological transformation of chlamydial reticulate bodies. Approximately 20 h following chlamydial infection, reticulate bodies begin condensation to form elementary bodies (27). Geometrical considerations suggest that the relative surface area of the reticulate bodies may be as much as 10 times that of the elementary bodies. During condensation, large amounts of outer membrane are probably shed as intracellular vesicles, similar to those demonstrated within chlamydial inclusions by Richmond and Stirling (26). Enriched with chlamydial LPS, these vesicles may fuse with the inclusion membrane; the LPS which has partitioned into the inclusion membrane can subsequently be incorporated into various membrane compartments of the host cell, including the plasma membrane, by several potential mechanisms. For example, the LPS in the inclusion membrane may be directed into a cytoplasmic lipid recycling pool. Alternatively, LPS-rich vesicles may arise on the cytoplasmic face of the inclusion membrane; such intracellular blebs may diffuse or be transported to the plasma membrane. Finally, LPS may bind to a variety of cellular proteins and lipoproteins, resulting in the formation of LPS-protein complexes (18), with subsequent intracellular sorting and translocation to the cell surface. This study cannot exclude the possibility that the LPS was transported through the plasma membrane and was observed in transit; however, biophysical considerations of the hydrophobic nature of chlamydial LPS would suggest that such a molecule, by virtue of its fatty acid constituents, should partition preferentially into a hydrophobic domain such as that provided by the phospholipid bilayer of the cellular plasma membrane.

The accumulation of LPS within cellular membranes decreases membrane fluidities (S. T. Karimi, R. A. Haak, and C. E. Wilde III, manuscript in preparation), possibly having significant consequences for the infected cell and host. Alterations in the physical properties of the membranes may affect the functions of various membrane systems such as endocytic processes and lysosome-endosome fusion. Simi-

able about 24 h following inoculation, and the apparent antigen density increased during the period of infection. The distribution of LPS antigen on bleblike structures on the surface of infected cells and on the proximal processes of neighboring uninfected cells suggested that the antigen could be shed from infected cells and adsorbed onto the membranes of adjacent cells. The use of monoclonal antibodies specific for MOMP failed to produce surface staining, indicating that the presence of LPS antigen on the surface was not caused by the release and readsoption of intact chlamydiae. Similar staining patterns were observed on unfixed and on aldehyde-fixed, air-dried cells, ruling out the contribution of fixation artifacts.

The appearance of staining on the surface of unfixed infected McCoy and L-929 cells indicated that the LPS antigen was accessible to antibody by virtue of its exposure on the surface of the plasma membrane. The lack of surface staining of unfixed infected HeLa cells does not preclude the incorporation of the LPS in the HeLa plasma membrane and is consistent with the hypothesis that membrane structural differences among the cell lines used may influence LPS antigen exposure. Such factors as membrane lipid and protein composition can affect the physical properties of the membrane fluidities (S. T. Karimi, R. A. Haak, and C. E. Wilde III, manuscript in preparation), possibly having significant consequences for the infected cell and host. Alterations in the physical properties of the membranes may affect the functions of various membrane systems such as endocytic processes and lysosome-endosome fusion.

TABLE 2. Ratios of LPS/MOMP reactivities in VSV preparations isolated from chlamydial-infected L-929 cells

<table>
<thead>
<tr>
<th>Sample</th>
<th>Reactivity(^a) of LPS/MOMP ratio(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L.\textsubscript{/434}/Bu infection:</td>
<td></td>
</tr>
<tr>
<td>Gradient pellet</td>
<td>6.7 × 10(^3) 3.0 × 10(^3) 2.2</td>
</tr>
<tr>
<td>VSV virion</td>
<td>6.1 × 10(^3) 1.1 × 10(^3) 5.5</td>
</tr>
<tr>
<td>Repurified VSV virion</td>
<td>2.0 × 10(^3) 0</td>
</tr>
<tr>
<td>Admixed VSV +</td>
<td>6.0 × 10(^3) 0</td>
</tr>
<tr>
<td>Elementary bodies</td>
<td></td>
</tr>
<tr>
<td>Purified elementary bodies</td>
<td>2.7</td>
</tr>
<tr>
<td>F/UW6/Cx infection:</td>
<td></td>
</tr>
<tr>
<td>Gradient pellet</td>
<td>7.6 × 10(^3) 2.7 × 10(^3) 2.8</td>
</tr>
<tr>
<td>VSV virion</td>
<td>2.1 × 10(^3) 1.2 × 10(^3) 17.5</td>
</tr>
<tr>
<td>Cell lysate</td>
<td>1.4 × 10(^3) 4.0 × 10(^3) 3.5</td>
</tr>
<tr>
<td>Purified elementary bodies</td>
<td>1.6</td>
</tr>
</tbody>
</table>

\(^a\) The total reactivity recovered is expressed as counts per minute of \(^{125}\)I-protein A bound upon treatment with antibody 47A2 (anti-LPS). antibody 1A2 (anti-L. MOMP), u antibody F221/6C2/2 (anti-F MOMP).

\(^b\) Counts per minute of \(^{125}\)I-protein A bound following treatment with 47A2 counts per minute of \(^{125}\)I-protein A bound following treatment with anti-MOMP antibodies.
larly, since the plasma membrane is the interface between the cell and its environment, perturbations of intercellular communication, immune recognition, and immune cytosis may occur. The efficiencies of these processes may be compromised by LPS-induced deviations from the optimal fluidities of cellular membrane structures. For example, preliminary studies have suggested that, despite binding to infected cell surfaces, 4/7A2 does not promote complement-mediated cytosis, perhaps because of a decrease in membrane fluidity sufficient to prevent insertion of the complement membrane attack complex.

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LITERATURE CITED


