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The Late Chlamydial Inclusion Membrane Is Not Derived from the Endocytic Pathway and Is Relatively Deficient in Host Proteins

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Chlamydiae are obligate intracellular parasites which multiply within infected cells in a membrane-bound structure termed an inclusion. Newly internalized bacteria are surrounded by host plasma membrane; however, the source of membrane for the expansion of the inclusion is unknown. To determine if the membrane for the mature inclusion was derived by fusion with cellular organelles, we stained infected cells with fluorescent or electron-dense markers specific for organelles and examined inclusions for those markers. We observed no evidence for the presence of endoplasmic reticulum, Golgi, late endosomal, or lysosomal proteins in the inclusion. These data suggest that the expansion of the inclusion membrane, beginning 24 h postinoculation, does not occur by the addition of host proteins resulting from either de novo host synthesis or by fusion with preexisting membranes. To determine the source of the expanding inclusion membrane, antibodies were produced against isolated membranes from *Chlamydia*-infected mouse cells. The antibodies were demonstrated to be solely against *Chlamydia*-specified proteins by both immunoprecipitation of [^{35}S]methionine-labeled extracts and Western blotting (immunoblotting). Techniques were used to semipermeabilize *Chlamydia*-infected cells without disrupting the permeability of the inclusion, allowing antibodies access to the outer surface of the inclusion membrane. Immunofluorescent staining demonstrated a ring-like fluorescence around inclusions in semipermeabilized cells, whereas Triton X-100-permeabilized cells showed staining throughout the inclusion. These studies demonstrate that the inclusion membrane is made up, in part, of *Chlamydia*-specified proteins and not of existing host membrane proteins.

Chlamydiae are obligate intracellular bacterial parasites which multiply within infected cells in a membrane-bounded structure (34). Infectious bacteria, termed elementary bodies (EB), are internalized by an endocytic event and are initially surrounded by the plasma membrane of the host cell (44). When the developing chlamydial microcolony is large enough to be seen by light microscopy, the structure is termed an inclusion. The inclusion membrane plays a key role in the survival of the parasite, allowing access to ATP (13) while physically separating the bacteria from cellular defense mechanisms (i.e., lysosomes [7, 8]). As bacteria multiply, the inclusion gains membrane to allow for the increase in volume. Despite its importance to the survival of the organism, little is known about the nature of the inclusion membrane.

Neither bacterial replication nor expansion of the inclusion is affected by cycloheximide-mediated inhibition of host protein synthesis (2). This observation indicates that the protein components of the inclusion membrane do not arise by de novo host protein synthesis. Therefore, the inclusion membrane is derived either from bacteria or from preexisting host membranes. Because chlamydiae enter cells by endocytosis (15, 16), we hypothesized that the source of the inclusion membrane may be part of the host endocytic apparatus. To examine this hypothesis, infected cells were incubated with ligands known to accumulate by endocytic activity, and the inclusion was examined for the presence of those ligands. Our results demonstrate that for both *Chlamydia psittaci* and *C. trachomatis*, the late inclusion is not part of the endocytic pathway.

Recent work by Hackstadt et al. (12) has demonstrated that a lipid component of the expanding inclusion is derived in part from the Golgi apparatus. Using lectins and antibodies targeted to specific organelles, we demonstrate that the inclusion membrane proteins are not derived from plasma membrane or selected internal membranes such as the Golgi apparatus or the endoplasmic reticulum (ER).

MATERIALS AND METHODS

Host cells. HeLa, CV-1, or MDBK cells were grown in antibiotic-free Eagle's minimal essential medium containing 5% heat-inactivated fetal calf serum. These host cells are readily infected by either *C. trachomatis* or *C. psittaci*. For experiments involving fluorescence microscopy, the cells were plated on glass coverslips (Fisher Scientific, Pittsburgh, Pa.). For experiments involving electron microscopy, the cells were grown on Permanox dishes (Miles Laboratories, Naperville, Ill.).

Infection. *C. trachomatis* serovar E or *C. psittaci* CAL-10 EB were prepared as described by Wyrick et al. (44), adjusted to a concentration of 10^{10} EB per ml, and stored at −70°C in 2× sucrose-phosphate buffer. Nonconfluent monolayers were incubated for 2 to 4 h with either *C. trachomatis* or *C. psittaci* EB at a multiplicity of infection of 200 to 400 EB per host cell. The cells were washed extensively and incubated in fresh medium for a further 16 to 72 h at 37°C at 5% CO_2.

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Permeabilization of cells. For studies using antibodies against host proteins or lectins, infected cells were washed with phosphate-buffered saline (PBS), fixed with 3.7% formaldehyde in PBS, and then washed with Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl [pH 7.6]). The fixed cells were permeabilized by incubation with 0.2% Triton X-100 in TBS for 5 min at room temperature. This procedure allows antibodies and lectins access to the interior of the chlamydial inclusion. For studies using antibodies against *Chlamydia*-specified proteins, we used a permeabilization technique which left the inclusion membrane intact. This procedure was a modification of the glass bead procedure of Fennell et al. (9). Infected cells, fixed with 3.7% formaldehyde in PBS, were washed with calcium-free Hanks balanced salt solution containing 0.6 M sucrose (pH 7.4). Glass beads (425 to 600 μm) were layered over host cells for 10 min at room temperature and then rinsed off with Hanks balanced salt solution-Ca^{2+} containing 0.6 M sucrose (pH 7.4).

Fluorescence microscopy. Host cells, infected for specified periods of time, were incubated with Lucifer yellow-CH (1 mg/ml; Aldrich, Milwaukee, Wis.) in minimal essential medium for periods ranging from 10 min to 2 h. The coverslips were washed with PBS, fixed by incubation in 3.7% formaldehyde in PBS for 10 min, and then washed with PBS. The coverslips were placed on glass slides, sealed with clear nail polish, and examined in a Zeiss fluorescence microscope.

In studies using antibodies or lectins, host cells permeabilized as described above were extensively washed in TBS. The antibodies were diluted in TBS containing 2 mg of bovine serum albumin per ml and were incubated with host cells for 1 h at room temperature. Host cells were washed and incubated for 1 h with the appropriate fluorescence-labeled secondary antibody at a 1/200 dilution. The coverslips were washed with TBS and after the last wash were mounted on microscope slides, using a mounting medium of glycerol-PBS with *p*-phenylenediamine (90% [wt/vol] glycerol, 0.5% [wt/vol] *p*-phenylenediamine [pH 8.5]) added to reduce fluorescence quenching. The coverslips were sealed with nail polish and examined in either a Zeiss fluorescence microscope, a Bio-Rad laser confocal microscope, or a Nikon inverted fluorescence microscope with a Zeiss 100× oil immersion objective.

Electron microscopy. Host cells that had been infected for 24 h were exposed to horseradish peroxidase (HRP; 1 mg/ml; Sigma Co., St. Louis, Mo.) or a transferrin (Tf)-HRP conjugate (10^{-8} M) for 1 h, extensively washed with cold PBS, and then fixed by using a modification of the procedure of Graham and Karnovsky (10). Briefly, monolayers were washed three times with cacodylate buffer (0.1 M sodium cacodylate, 0.1 M sucrose, 0.01% [wt/vol] $CaCl_2$ [pH 7.2]). The monolayers of host cells were then incubated with 2.0% glutaraldehyde–0.5% paraformaldehyde in cacodylate buffer for 15 min at 37°C. The host cells were washed twice with cacodylate buffer and then twice with STE (10 mM Tris, 5 mM EDTA, 0.6 M sucrose [pH 7.2]) and subsequently incubated with 0.5 mg of diaminobenzidine (Sigma) per ml–0.01% H_2O_2 in Tris buffer for 15 min at 37°C. HeLa cells were washed twice with STE and twice with cacodylate buffer and then incubated with 1.0% OsO_4 in cacodylate buffer for 15 min at room temperature. The cells were then washed four times with cacodylate buffer, en bloc stained with 5% uranyl acetate (in 70% ethanol), and dehydrated in ethanol. The samples were placed in Eponate (Pella; Tustin, Calif.); thin sections were cut with a diamond knife and were viewed in a JEOL 100S electron microscope.

Virus constructs. Viral constructs were generously provided by M. G. Roth (University of Texas Medical Center, Dallas). The construct SVEHA^{sec} (33) contains the coding sequence for the influenza virus hemagglutinin (HA) in which the membrane-spanning domain had been removed. Following incubation with a rabbit anti-HA immunoglobulin G (IgG) antibody (1/200 dilution) and fluorescent second antibody (fluorescein isothiocyanate [FITC]-labeled goat anti-rabbit antisera), the truncated glycoprotein can be detected within the lumen of the ER and Golgi apparatus. A second virus construct, SVHABAR, specifies the synthesis of a hybrid protein, the extracellular domain of influenza virus HA fused to the transmembrane domain of the turkey β-adrenergic receptor. The product of this construct is inserted into the ER membrane but does not get transported to the Golgi apparatus.

Antisera and lectins. Rabbit antiserum was prepared against purified EB of *C. psittaci* as described by Hodinka and Wyrick (16). Rabbit polyclonal anti-human Tf receptor antibody was a generous gift from Paul E. Seligman. Rabbit polyclonal anti-bovine mannose 6-phosphate receptor antibody was a generous gift from Stuart Kornfeld. The monoclonal antibody directed against human lysosomal membrane proteins (Lamp-1 and Lamp-2) developed by Manes et al. (26) was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology in Molecular Sciences, Johns Hopkins University School of Medicine, and the Department of Biological Sciences, University of Iowa. Fluorescein-conjugated goat anti-rabbit immunoglobulin and mouse anti-major outer membrane protein antibody were purchased from ICN ImmunoBiologicals (Lisle, Ill.). Fluorescent lectins were the generous gift of W. Samlowski (University of Utah). All experiments (different antibodies or lectins) were done at least three times with different infections. Conservatively, several hundred infected cells were examined for each experiment.

A mouse monoclonal antibody directed against chlamydiae, goat anti-mouse alkaline phosphatase-conjugated antibody, rabbit anti-mouse IgG antibody, and fluorescein-conjugated goat anti-rabbit immunoglobulin were purchased from ICN ImmunoBiologicals, Inc. (Lisle, Ill.). According to the vendor, the antigen recognized by the mouse monoclonal antibody directed against chlamydiae is a heat-stable chlamydial component. The antibody has no cross-reactivity with lipopolysaccharide from other gram-negative bacteria and reacts with *C. psittaci*, *C. psittaci*, and *C. trachomatis.*

A polyclonal antiserum directed against membrane from *Chlamydia*-infected mouse cells was prepared in the following manner. Mouse cells (3T3) of BALB/c origin were infected with *C. psittaci*. After 36 h in culture, the infected cells were washed with PBS, removed from the monolayer by use of a rubber policeman, harvested by centrifugation at 400 × *g* for 10 min, and resuspended in a buffer containing 0.25 M sucrose and 10 mM Tris (pH 7.2). Disruption of the cells was accomplished with a Dounce homogenizer, and the homogenate was centrifuged at 400 × *g* for 10 min to remove intact host cells and nuclei; the remaining homogenate was centrifuged at 10,000 × *g* for 30 min. The supernatant was discarded, and the pellet (containing infected HeLa cell membrane and chlamydial membrane) was resuspended in the sucrose-Tris buffer. This suspension was mixed with Freund's complete adjuvant and used to intraperitoneally immunize BALB/c mice.

Electrophoresis and Western blotting (immunoblotting). Infected and noninfected HeLa cells were extracted with radioimmunoprecipitation assay buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 50 mM Tris [pH 7.5]) at 0°C for 30 min. Extracts (20 μg of protein per lane) were loaded on an SDS–10% gel. Following electrophoresis, gels were electrophoretically transferred to nitrocellulose blotting membrane (Costar, Cambridge, Mass.). The nitrocellulose filter was incubated for 1 h at room temperature in blocking buffer (5% nonfat dried milk, 0.1% Tween 20, 10 mM Tris-HCl, 0.9% NaCl [pH 8.0]). Following several washes in T-TBS (0.1% Tween 20, 10 mM Tris-HCl, 0.9% NaCl [pH 8.0]), the blotting membrane was incubated in diluted (1/1,000) mouse anti-chlamydial membrane antibody or preimmune serum in T-TBS for 1 h (37°C), rinsed with T-TBS, and incubated in diluted (1/5,000) goat anti-mouse alkaline phosphatase-conjugated antibody. After several rinses with T-TBS, blots were developed in the presence of bromochloroindolyl phosphate-nitroblue tetrazolium.

Immunoprecipitation and electrophoresis. Infected and noninfected HeLa cells were incubated for 2 days in ^{35}S-labeled methionine prior to extraction in a radioimmunoprecipitation assay buffer for 30 min at 0°C. Extracts were precleared with an equal volume of 20% Pansorbin cells (Calbiochem, San Diego, Calif.) for 30 min on ice. After centrifugation (4,000 × *g*, 5 min), the supernatant was incubated with mouse anti-chlamydial membrane antibody or preimmune serum (1 h, 0°C), then with the rabbit anti-mouse IgG (1 h, 0°C), and finally with Pansorbin cells (1 h, 0°C). The immune complex on Pansorbin cells was rinsed by centrifugation (10,000 × *g*, 1 min). Following electrophoresis on an SDS–10% running gel, the gel was stained with Coomassie blue, destained, soaked in Amplify (Amersham International, Amersham, England), and dried. Autoradiographic film was placed directly on the gel and exposed at −70°C.

RESULTS

Distribution of internalized ligands in infected host cells. The inclusion in HeLa cells infected with *C. trachomatis* or *C. psittaci* was large enough to be recognized by phase-contrast microscopy 24 h after infection (Fig. 1A). Each inclusion contained a number of bacteria which were easily stained by antichlamydial antibodies in Triton X-100-permeabilized host cells (data not shown). Previous studies demonstrated that internalized live chlamydiae are not found within lysosomes, whereas internalized heat-killed chlamydiae can be found within this organelle (8). We hypothesized that perhaps the inclusion resulted from the live bacteria interfering with normal intracellular vesicular traffic, and thus the inclusion represented an intermediate stage along the endocytic pathway. To determine if the inclusion was derived from the endocytic pathway, infected cells were incubated with ligands which accumulate by endocytic activity. Lucifer yellow, an intensely fluorescent molecule, is internalized in host cells by fluid-phase endocytosis and distributes throughout the endocytic apparatus, staining both lysosomes and endosomes. Host cells, which had been infected with *C. trachomatis* for 20 h, were incubated with lucifer yellow (1.0 mg/ml) for times ranging from minutes to hours and examined for the distribution of fluorescence. Heavily infected cells appeared to accumulate lucifer yellow to the same extent as uninfected cells. Infected cells contained fluorescent vesicles of widely differing sizes, reflecting the presence of lucifer yellow in both endosomes and lysosomes. The chlamydial inclusion stood out as a black hole in a highly fluorescent background, indicating that the dye is excluded from the inclusion (Fig. 1B). There was no evidence of fluo-

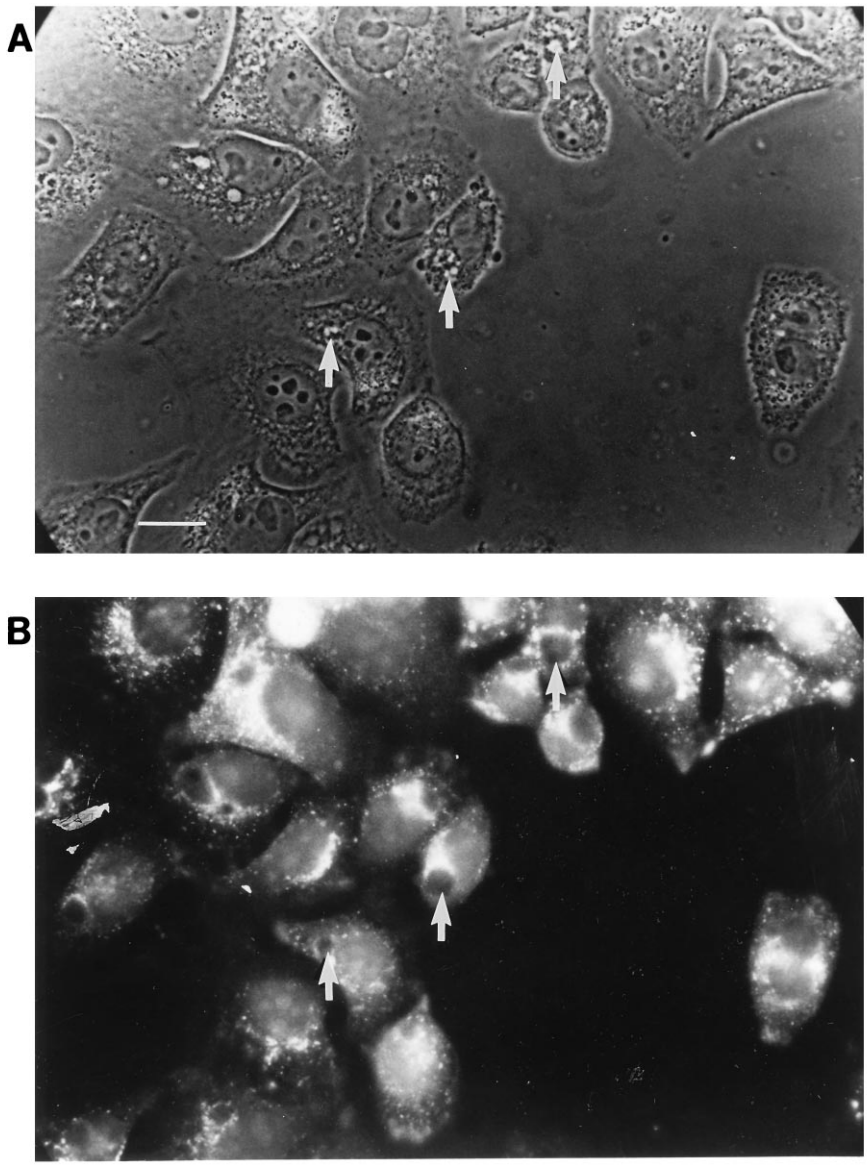

FIG. 1. Accumulation of lucifer yellow in *Chlamydia*-infected cells. HeLa cells at 16 to 24 h after infection with *C. trachomatis* were incubated with Lucifer Yellow-CH (1 mg/ml) for 1 h. (A) Phase-contrast images in which inclusions can be seen as large vacuoles. A number of them are marked by arrowheads. (B) The corresponding fluorescent image. Bar, 20 μm.

rescent molecules in the inclusion even after a 2-h incubation with lucifer yellow (data not shown).

High concentrations of lucifer yellow in other organelles may have obscured visualization of the marker in inclusions. To examine this possibility, we utilized HRP as a fluid-phase marker and examined its distribution in infected cells by electron microscopy. In HeLa cells infected with *C. trachomatis* for 24 h and then incubated for 1 h with HRP, peroxidase activity was detected in small, amorphously shaped compartments and in large vesicular compartments, presumably lysosomes (Fig. 2). No activity was found in the inclusion, again suggesting that fluid-phase markers do not gain access to the inclusion.

Accumulation of fluid-phase markers predominately involves the part of the endocytic apparatus which is directed to the lysosome. Markers such as lucifer yellow or HRP are found in very low concentration in vesicles which are destined to be recycled to the cell surface. The inclusion could be part of the recycling endocytic pathway and thus would not be expected to accumulate those markers. To test this hypothesis, we used a Tf-HRP conjugate as a marker for the recycling portion of the endocytic apparatus. In contrast to most other recycling receptors, Tf remains associated with its receptor within the recycling pathway (4, 24). Incubation of either infected or noninfected HeLa cells simultaneously with Tf-HRP and excess Tf prevented uptake of the conjugate (data not shown). This observation confirms earlier biochemical studies demonstrating that the conjugate is internalized as a result of binding to the Tf receptor (1). Infected cells were incubated with the Tf-HRP conjugate for 60 min, processed for electron microscopy, and examined for the presence of peroxidase activity (Fig. 3). On a qualitative level, it appeared that control host cells and infected host cells accumulated similar amounts of the Tf-HRP conjugate. Peroxidase activity was not found in large vesicular structures, indicating that most of the conjugate

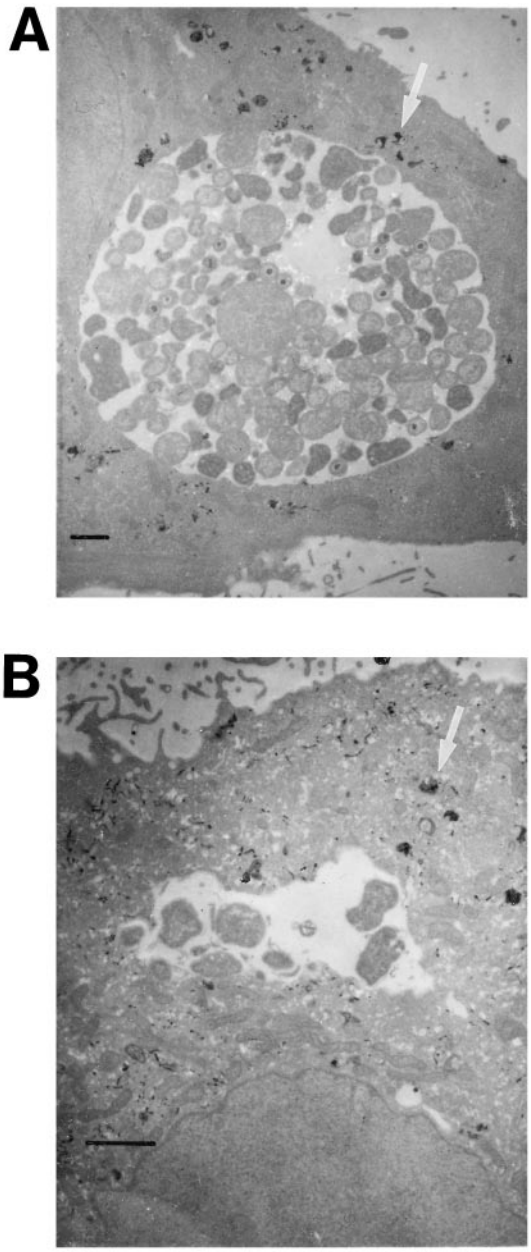

FIG. 2. Accumulation of HRP in *Chlamydia*-infected cells. HeLa cells at 20 h after infection with *C. trachomatis* were exposed to HRP (1 mg/ml) for 1 h. The HeLa cells were extensively washed with cold PBS, fixed, and stained for peroxidase activity by using diaminobenzidine. Panels A and B are images of inclusions in different cells infected with *C. trachomatis*. Arrowheads indicate HRP. Bar, 1 μm.

entered the host cell by the endocytic pathway and not by the fluid-phase pinocytosis. Peroxidase activity was commonly found in small elongated vesicles which were clustered around the inclusion. The contents of these vesicles were clearly separated from that of the inclusion by the membranes of the inclusion and the endosome. No peroxidase activity was seen on the inside of the inclusion. Previous studies demonstrate that endocytic markers are present in the newly internalized bacterial inclusion. These results indicate that the expanding inclusion membrane is not from the endocytic pathway.

Distribution of Tf receptors in infected cells. The studies described above indicated that the 20- to 24-h chlamydial inclusion is not part of the endocytic apparatus. The question remained as to the source of membrane for the expanding inclusion. Other studies have suggested the existence of recycling pathways different from those for receptor-mediated endocytosis (38). The inclusion might be composed of plasma membrane obtained from alternate recycling pathways. To test this, we examined the distribution of Tf receptors in infected cells. Infected cells were fixed, permeabilized by using Triton X-100, incubated with antibody against the Tf receptor, and examined by confocal fluorescence microscopy. This approach provided evidence that Tf receptors were not part of the inclusion but were in vesicles surrounding the inclusion membrane. The images in Fig. 4A and B are two optical sections of an infected cell. The inclusion appears as a dark circle sur-

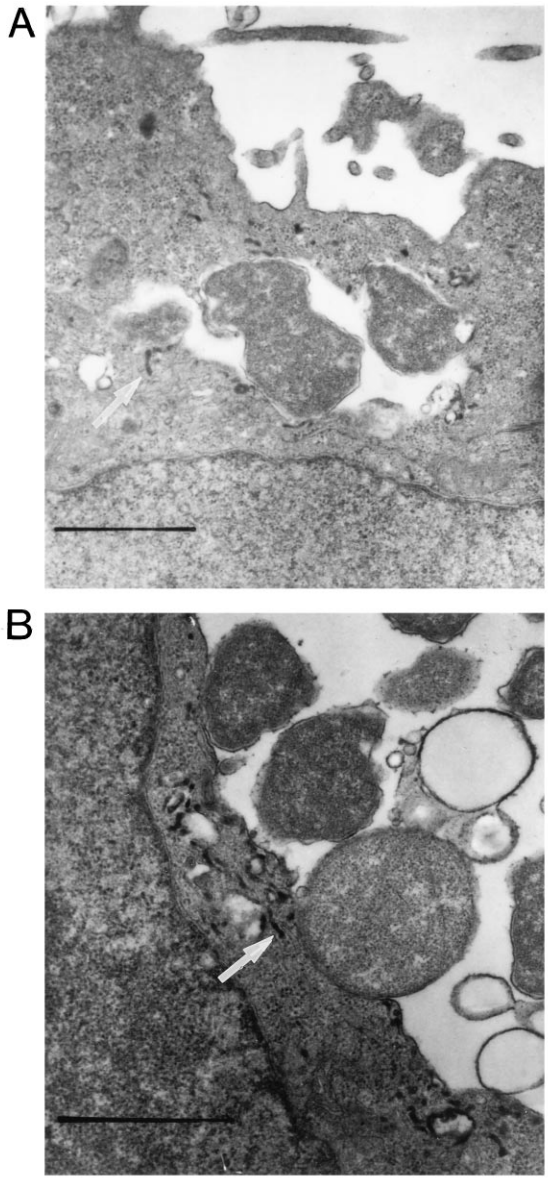

FIG. 3. Distribution of Tf-HRP in *Chlamydia*-infected cells. HeLa cells at 24 h after infection with *C. trachomatis* were incubated with 10^{-8} M Tf-HRP for 1 h at 37°C. At the end of the incubation period, the infected cells were placed on ice, washed with cold buffer, and prepared for electron microscopy. Panels A and B are images of inclusions in different HeLa cells infected with *C. trachomatis*. Arrowheads indicate Tf-HRP. Bar, 1 μm.

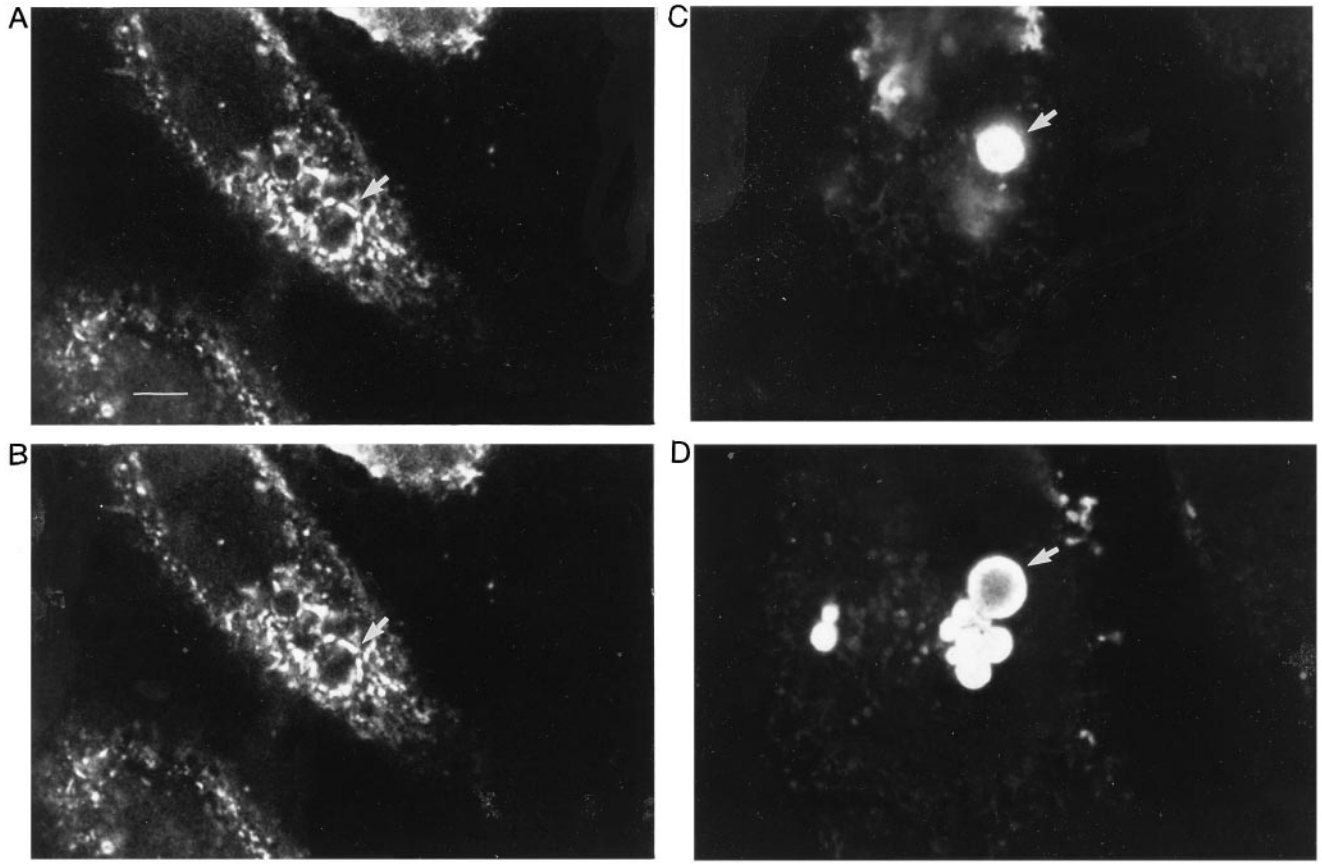

FIG. 4. Distribution of Tf receptors in *Chlamydia*-infected cells as examined by confocal microscopy. HeLa cells at 48 h after infection with *C. psittaci* were fixed, permeabilized with 0.2% Triton X-100 in TBS, and stained with either a rabbit anti-human Tf receptor antibody or a rabbit anti-*C. psittaci* antibody. The cells were then stained with a FITC-labeled goat anti-rabbit antibody. The samples were viewed by confocal fluorescence microscopy. The images presented are part of a consecutive series. Arrowheads mark a number of inclusions. (A and B) Cells stained with rabbit anti-human Tf receptor; (C and D) cells stained with rabbit anti-*C. psittaci* antibody. Bar, 10 μm.

rounded by bright vesicles which extend incompletely around the inclusion. For comparison purposes, confocal images of infected cells stained with a rabbit antibody directed against *C. psittaci* are shown in Fig. 4C and D. In these images, the distribution of fluorescence is smooth and continuous. These results suggest that the inclusion does not contain detectable amounts of Tf receptors. While we have not quantified the distribution of Tf receptor-containing endosomes within infected cells, our observations indicate that these organelles appear to cluster around the inclusion.

Staining of infected cells with plant lectins. One conclusion from the results presented above is that the chlamydial inclusion does not grow by addition of host plasma membrane or endocytic proteins. It is possible, however, that Tf receptors are specifically excluded from the inclusion membrane while other plasma membrane markers are not. To address this issue, we used fluorescent plant lectins to examine the inclusion for the presence of carbohydrates. Lectin staining has been used to identify the presence of carbohydrate-containing molecules on plasma membranes and other intracellular organelles (14, 42). Nonpermeabilized infected cells incubated with either concanavalin A or *Triticum vulgaris* agglutinin, both of which are lectins specific for terminal mannose residues, showed only circumferential plasma membrane staining (data not shown). In comparison, Triton X-100-permeabilized MDBK cells showed a large increase in staining of different-size vesicular structures (Fig. 5A). Identical results were obtained with lectins such as *Lotus tetragonolobus* agglutinin (Fig. 5B), specific for fucose, and *Lens culinaris* lectin (Fig. 5C), specific for glucose terminal carbohydrates. Simultaneous additions of the appropriate sugar (methyl mannose, galactose, or fucose; 0.1 M) completely prevented fluorescent staining of intracellular organelles. The 36-h *C. psittaci* inclusions again were easily recognizable as dark, unstained structures. When fluorescence was observed near or adjacent to the inclusions, a discontinuous, patchy distribution was observed. This is not the pattern that we would predict if the lectin-staining proteins were part of the inclusion. In that instance, we would expect fluorescence associated with the inclusions to be continuous and circumferential. These results indicate that the chlamydial inclusion membrane does not contain a high density of carbohydrates molecules. Since most ER and Golgi proteins are glycosylated, this result suggests that these membrane systems do not contribute proteins to the inclusion membrane.

Staining of infected cells with antibodies specific for the ER or Golgi apparatus. To further examine the foregoing conclusion, a number of different antibodies specific for ER proteins were used to determine if the chlamydial inclusion was composed of elements derived from the ER and/or Golgi apparatus. Most of the antibodies available to us did not allow clear resolution of these structures in either infected or noninfected host cells. To circumvent this problem, we took advantage of

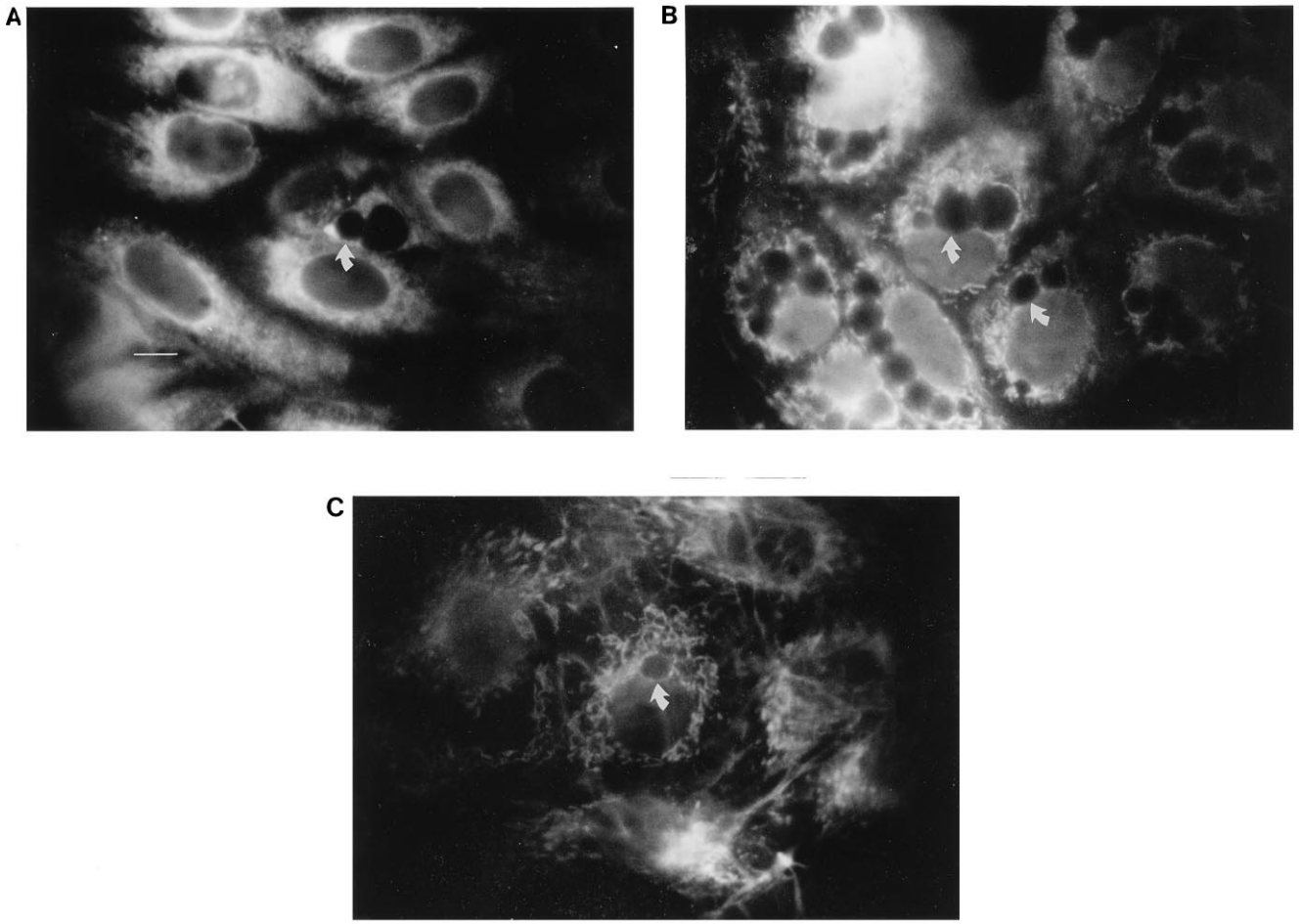

FIG. 5. Distribution of FITC-conjugated lectins in permeabilized *Chlamydia*-infected cells. MDBK cells at 36 h after infection with *C. psittaci* were permeabilized with 0.2% Triton X-100 in TBS and then incubated with either concanavalin A-FITC (A), *Lotus tetragonobolus* agglutin-FITC (B), or *Lens culinaris* lectin-FITC (C) Arrowheads mark a number of inclusions. Bar, 10 μm.

viral constructs that directed the synthesis of virus-specific proteins which accumulated in the ER and/or Golgi apparatus. We used a simian virus 40 (SV40) viral construct containing an influenza virus HA protein in which the membrane-spanning domain was removed ($SVEHA^{sec}$) (33). This truncated HA is secreted and acts as a marker for the lumen of the ER and Golgi apparatus. A second SV40 construct used (SVHABAR) contained an insert in which the ectodomain of the HA was fused to the transmembrane domain of the β-adrenergic receptor. The expressed membrane protein is not transported out of the ER and thus is a marker for ER membranes. The concentrations of these proteins in host cells were high enough that they permitted both Golgi and ER compartments to be clearly resolved by indirect immunofluorescence. Simultaneous infection of cells with the viral constructs and *C. psittaci* did not affect either production of viral proteins or multiplication of chlamydiae. Regardless of the viral construct used to infect cells, neither inclusions nor inclusion membranes were stained by HA-specific antibodies. Images obtained by conventional fluorescence microscopy (Fig. 6) exhibit vivid staining of the ER or Golgi apparatus. There is no evidence that influenza virus-specific antigens are located within the inclusion, as expected for a content marker (Fig. 6A), or around the inclusion (Fig. 6B), as expected for a membrane marker. Fluorescence observed near the inclusion did not appear to be either continuous within the plane of focus or contiguous within different planes of foci (data not shown).

To confirm the observation that Golgi markers do not comprise the inclusion, infected cells were stained with an antibody directed against the cation-dependent mannose 6-phosphate receptor. While this molecule can be found on the host cell surface, its major location is in the Golgi apparatus and the late endosome. The receptor's major intracellular function is to transfer mannose 6-phosphate-containing proteins from the Golgi apparatus to the late endosome and finally to the lysosome. On the cell surface, it functions as a receptor for insulin-like growth factor II (25). Immunofluorescence revealed vesicles (Fig. 7A) which, in heavily infected cells, were closely adjacent to the inclusion. Examination of different focal planes through an infected cell demonstrated that the staining pattern was not circumferential within a plane of focus and was discontinuous between planes. These results further indicate that while the stained vesicular material is close to an inclusion, it is not part of the inclusion.

Mannose 6-phosphate receptors normally are found in the highest concentration in the Golgi apparatus and in late endosomes. Our studies, and those of Eissenberg and Wyrick (7), demonstrate no fusion of inclusion with preexisting lysosomes. This result was based on the use of content markers, in our case HRP and lucifer yellow. We have further demonstrated

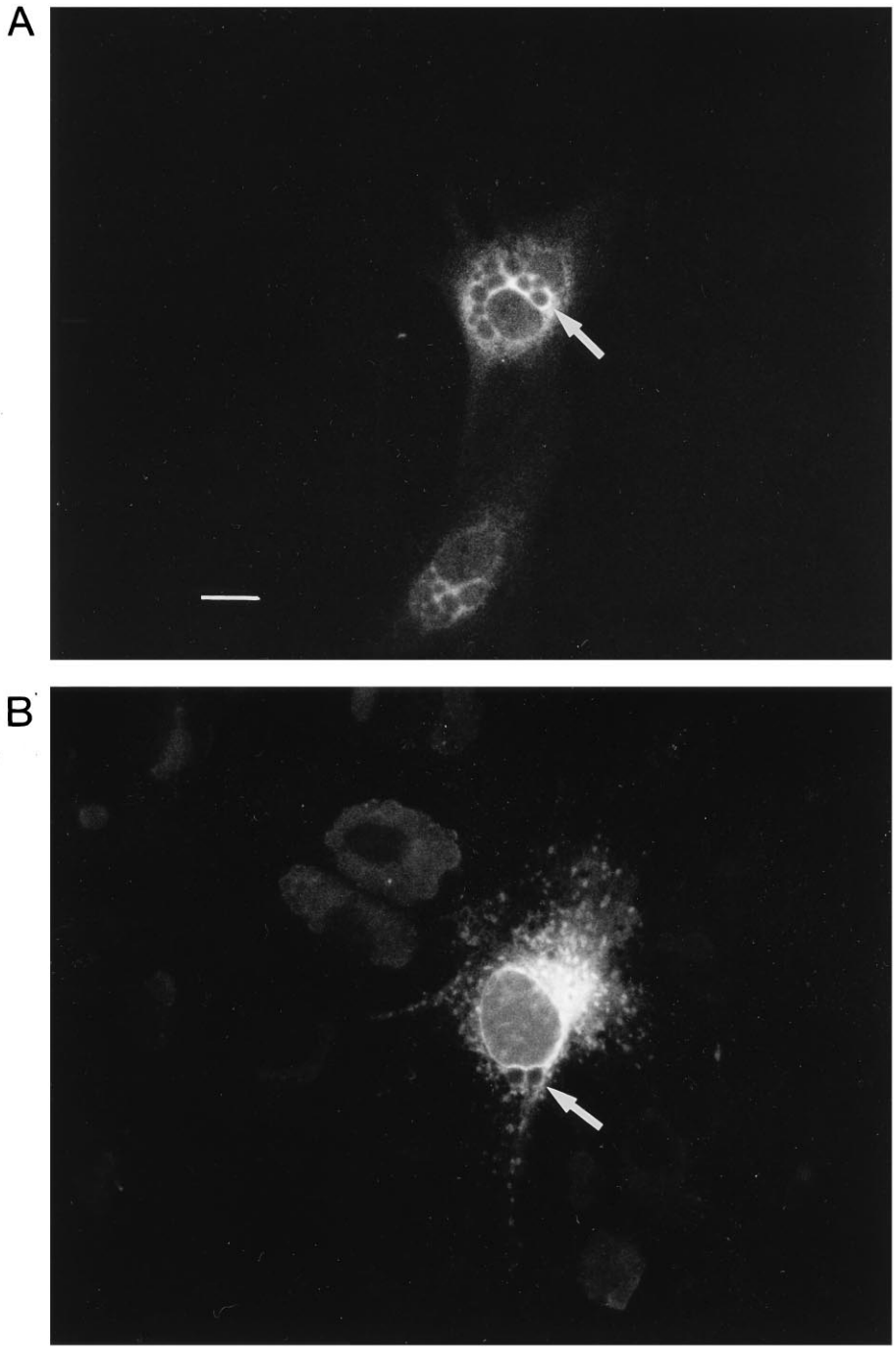

FIG. 6. Distribution of genetically engineered influenza virus HA in *Chlamydia*-infected cells. CV-1 cells were simultaneously infected with *C. psittaci* and either one of two SV40 constructs: SVEHA^{sec}, which specified a secreted influenza virus HA (A), or SVHABAR, in which the extracellular domain of the influenza virus HA had fused to the transmembrane domain of the turkey β-adrenergic receptor (B). At 44 h postinfection, cells were permeabilized with Triton X-100 and stained with a rabbit anti-Influenza virus IgG followed by an FITC-labeled goat anti-rabbit antiserum. The cells were examined by conventional fluorescence microscopy. Arrowheads mark inclusions. Bar, 20 μm.

that the inclusion membrane does not contain lysosomal or late endosomal membrane proteins by using infected cells which were stained with an antibody against a lysosomal glycoprotein (Lamp-1 or Lamp-2), which is enriched in lysosomes and late endosomes. Examination of infected cells revealed intense staining of vesicular structures with antibody to either Lamp-1 (data not shown) or Lamp-2 (Fig. 7B). By either conventional or confocal microscopy, these organelles were separated from inclusions, providing evidence that the lysosomal glycoprotein (Lamp) is not contained within the inclusion membrane, and thus there is no fusion between lysosomes or endosomes and mature inclusions.

The inclusion membrane may be composed of *Chlamydia*-specified proteins. Matsumoto (27) has presented micrographs

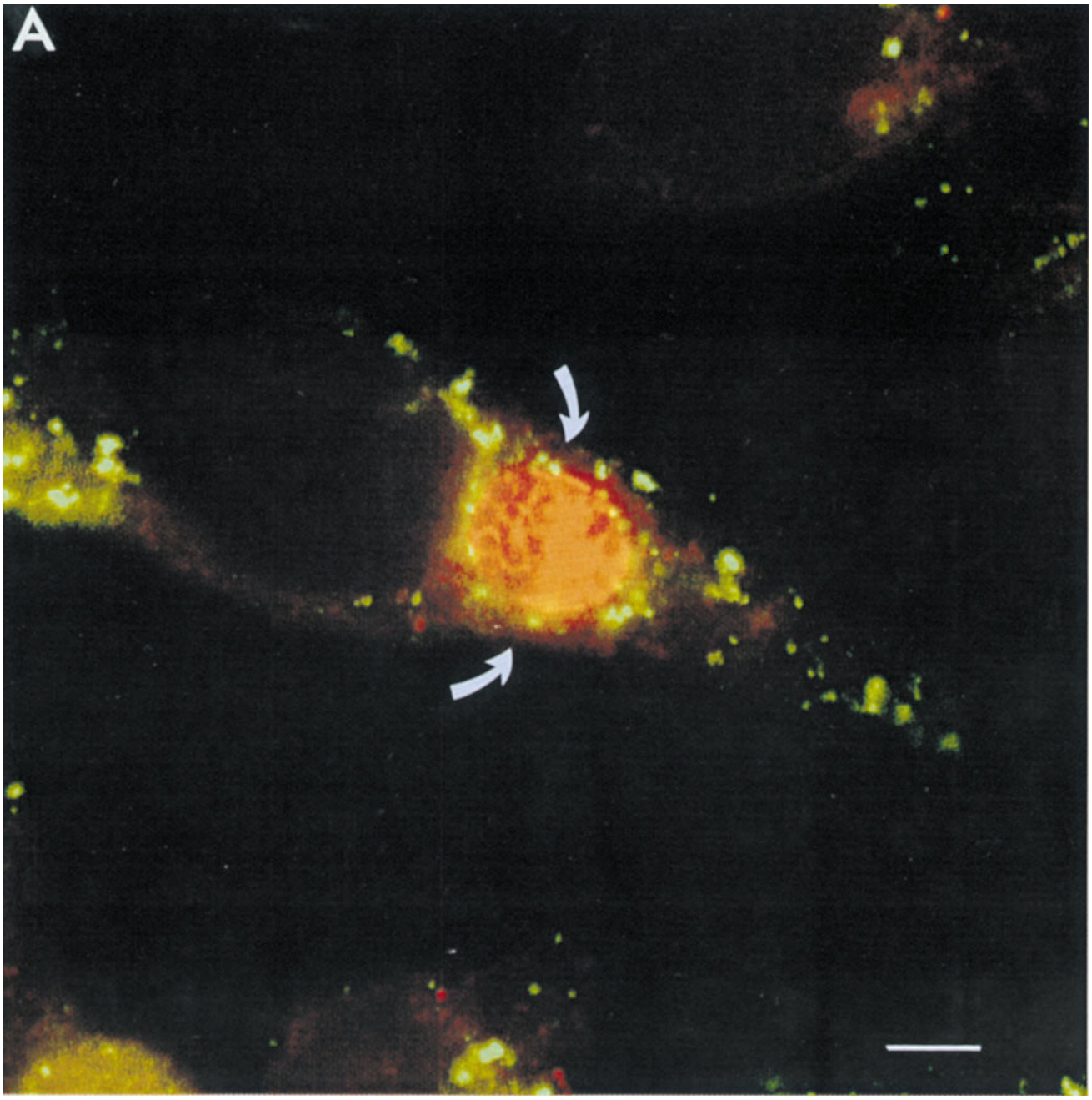

FIG. 7. (A) Distribution of mannose 6-phosphate receptors in *Chlamydia*-infected cells. HeLa cells at 48 h after infection with *C. trachomatis* were permeabilized with 0.2% Triton X-100 in TBS and labeled with either rabbit anti-rat mannose 6-phosphate receptor antibody or mouse antichlamydia antibody. The infected cells were then stained with an FITC-labeled goat anti-rabbit antibody or Texas red-label goat anti-mouse antibody. The samples were viewed by Oncor imaging fluorescence microscopy. Bar, 10 μm. (B) Distribution of lysosomal membrane glycoproteins in *Chlamydia*-infected cells. HeLa cells at 48 h after infection with *C. trachomatis* were permeabilized with 0.2% Triton X-100 in TBS and labeled with mouse anti-human Lamp-2 antibody. The infected cells were then stained with an FITC-labeled goat anti-mouse antibody. The samples were viewed by Oncor imaging fluorescence microscopy. Bar, 10 μm.

which he interpreted as demonstrating chlamydial structures which project into and through the inclusion membrane. This observation suggests that the growing inclusion membrane may contain membranous material derived from the bacteria. To test this hypothesis, we generated an antiserum that was specific to the membrane from 3T3 cells infected with *C. psittaci* and used this antiserum to stain chlamydial infected cells.

Properties of the antichlamydial serum. Cultured 3T3 cells (derived from BALB/c mice) were infected with *C. psittaci*. The infection was allowed to proceed for 24 h, and then the cells were homogenized and a membrane fraction was obtained by differential centrifugation (see Materials and Methods). The membranes were used to immunize BALB/c mice, and a mouse antichlamydial antiserum was obtained. Using immunofluorescence, we demonstrated that the antiserum stained only structures in infected cells; these stained structures were

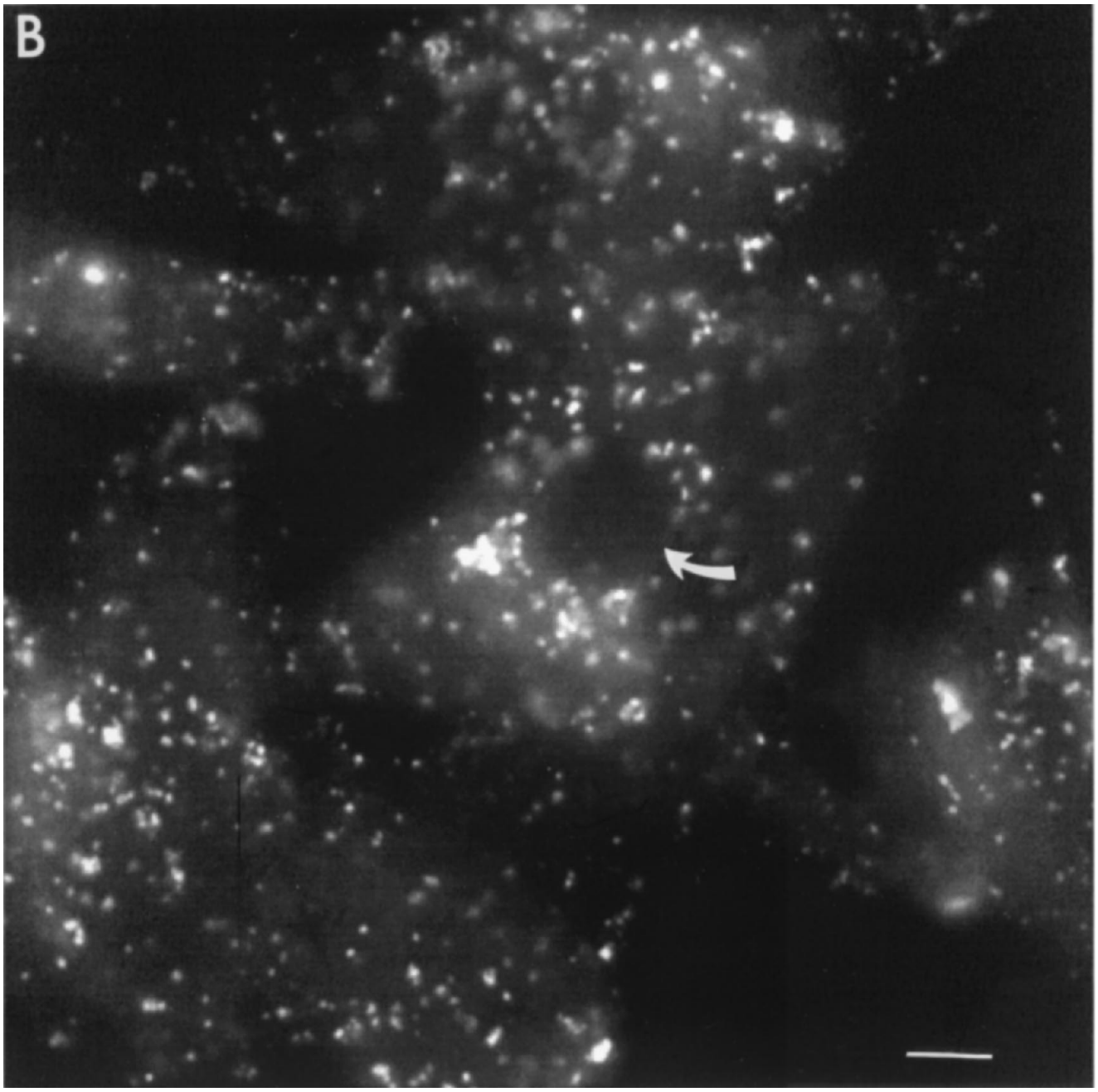

FIG. 7—*Continued.*

not observed when preimmune serum was used (see below). Western blot analysis was used to compare extracts of infected and noninfected HeLa cells. In infected mouse cells (data not shown) or HeLa cells (Fig. 8), the mouse antichlamydial membrane antibody detected a number of proteins. Proteins of 29, 57, and 71 kDa were particularly predominant in infected HeLa cell extracts probed with mouse anti-chlamydial membrane antibody followed by goat anti-mouse alkaline phosphatase-conjugated antibody. These bands were not observed in infected cells in assays using preimmune serum (Fig. 8, lane 3). These bands were observed in infected cells that had been incubated with cycloheximide (data not shown). Only the 57-kDa protein was seen in noninfected cell extracts. The membrane isolation used to generate antibodies is not specific for inclusion membranes. Therefore, one would expect to see some bands in noninfected cell extracts. However, as observed, some unique bands were identified in the infected cell extracts by using the antimembrane antiserum. These may be of host origin, or they may be *Chlamydia*-specific antigens.

To confirm that this mouse antichlamydial antiserum recognized *Chlamydia*-specified antigens, HeLa cells were labeled with [^{35}S]methionine and extracts were subjected to immunoprecipitation. This was done because epitopes may be denatured during the Western blotting procedure and thus would no longer be recognized by antibodies. Both infected and noninfected cells were incubated with [^{35}S]methionine for times

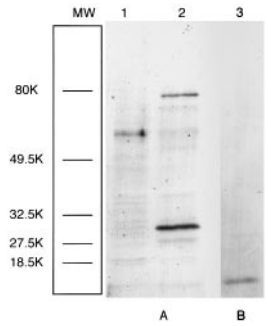

FIG. 8. Western blot analysis of infected and noninfected HeLa cell extracts. The blot in panel A was probed with mouse anti-chlamydial membrane antiserum followed by goat anti-mouse alkaline phosphatase-conjugated antibody. The blot in panel B was probed with preimmune serum followed by goat anti-mouse alkaline phosphatase-conjugated antibody. Lanes: 1, noninfected HeLa cell extract; 2 and 3, infected HeLa cell extract.

ranging from 1 to 48 h. In the case of infected cells, the labeling period was started approximately midway through the developmental cycle (24 h). Extracts of labeled cells (infected and noninfected) were prepared and immunoprecipitated, and the distribution of radioactivity was examined by SDS-polyacrylamide gel electrophoresis. We found labeled proteins of 42 and 52 kDa that were precipitated upon addition of the *Staphylococcus* protein A vehicle (Pansorbin). These bands were found in extracts from infected and noninfected cells in assays using preimmune serum, in the absence of antiserum, and even after attempts to preclear the extracts with *Staphylococcus* protein A. Addition of the polyclonal antiserum (against chlamydial membrane) to infected HeLa cell extracts resulted in a preferential precipitation of proteins of approximately 57 and 39 kDa (Fig. 9A). These bands were seen in extracts from infected cells that had been labeled in the presence or absence of cycloheximide (data not shown), indicating that these proteins were not host proteins induced as a result of chlamydial infection. We also compared the distribution of ^{35}S radioactivity in immunoprecipitates obtained by using the mouse antichlamydial membrane antiserum with a rabbit polyclonal antiserum made against purified *C. psittaci* (16) (Fig. 9B). While the relative amounts of radioactive protein precipitated were different, similar bands (57, 42, and 39 kDa) were found with both antisera (compare Fig. 9A and B).

The possibility exists that some of the epitopes recognized by the antiserum are either lipid or carbohydrate in nature and that these epitopes can modify host proteins by covalent attachment. To test this possibility, HeLa cells were incubated with [^{35}S]methionine for 48 h before infection. The HeLa cells were washed free of radiolabel, incubated in the presence of nonradioactive methionine, and infected in the presence of cycloheximide (1 μg/ml). After 36 h of infection, extracts were made and incubated with the mouse anti-chlamydial membrane antiserum, and immunoprecipitates were examined for radioactivity. The 42-kDa band was precipitated simply by addition of rabbit sera and Pansorbin cells. No other bands were observed, even after long development times (data not shown). These results indicate that the mouse antiserum is directed against *Chlamydia*-specific proteins (29- and 71-kDa bands detected by Western blot analysis and 39-, 42-, and 52-kDa bands detected by immunoprecipitation) and not host proteins modified by *Chlamydia*-specified products.

Staining of *Chlamydia*-infected cells. To determine if the inclusion membrane was composed of chlamydial proteins, infected cells were permeabilized by Triton X-100 and stained with the rabbit antichlamydial antibody. Noninfected cells permeabilized by Triton X-100 showed no staining with the rabbit antiserum. In infected cells, however, the staining was so bright that even with the confocal microscope we could not resolve the inclusion membrane from the contents of the inclusion (data not shown). On the basis of these observations, we took an alternate approach in which we selectively permeabilized cells under conditions which maintained the integrity of the inclusion. The cells were then stained with the antiserum, and the inclusion was examined for immunofluorescence. Cells were incubated with glass beads which adhere to and remove pieces of plasma membrane (9). Permeabilization of the plasma membrane and perhaps removal of some cytoskeletal elements resulted in lysis of the inclusion (data not shown). This lysis appeared to be the result of a difference in osmotic pressure between the inclusion and the medium, since lysis of inclusions could be prevented by the addition of 0.6 M sucrose.

Cells infected with *C. psittaci* were permeabilized and then incubated with either of two antichlamydial polyclonal antibodies (rabbit antiserum or mouse anti-chlamydial membrane antiserum). Secondary antibodies with a FITC label were used to visualize the staining patterns of intact and permeabilized inclusion. Semipermeabilized cells with intact inclusions demonstrated a ring-like fluorescence around the inclusion (Fig. 10A). In each optical section, the fluorescence was contiguous and restricted to the periphery of the structure (Fig. 10B). The center of the structure was dark, and no individual bacteria were detected. These results can be compared with those for infected cells permeabilized with Triton X-100 and stained with the mouse anti-chlamydial membrane sera. Under conditions in which the inclusion was permeabilized with Triton X-100, individual bacteria were readily detected (Fig. 10C). These observations suggest that in infected cells selectively permeabilized by the glass bead method, the antibody did not gain access to the interior of the inclusion but did stain the outer membrane of the inclusion.

Since membrane of host cells infected with *C. psittaci* was used to generate the antiserum, the possibility exists that the antiserum recognizes a lipid epitope. To examine this possibility, infected cells were selectively permeabilized, and antibody was added to label the inclusion membrane. The cells were then incubated with Triton X-100 prior to addition of the fluorescent second antibody. The rationale was that Triton X-100, by extracting lipids, would remove any lipids bound to

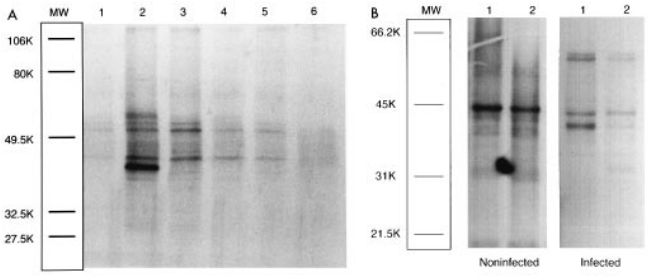

FIG. 9. Immunoprecipitation of infected and noninfected HeLa cell extracts. (A) Proteins were precipitated from infected (lanes 2, 4, and 6) and noninfected (lanes 1, 3, and 5) HeLa cell extracts following incubation with antibodies and/or Pansorbin cells. Lanes: 1 and 2, cell incubated with mouse antichlamydial membrane antiserum, rabbit anti-mouse antibody, and Pansorbin; 3 and 4, cells incubated with preimmune mouse serum, rabbit anti-mouse antibody, and Pansorbin; 5 and 6, cells incubated with Pansorbin. (B) Proteins were precipitated from noninfected and infected HeLa cell extracts following incubation with mouse anti-chlamydial membrane antiserum (lane 1) and rabbit anti-*C. psittaci* antibody (lane 2).

antibody. This procedure, however, did not affect the circumferential staining pattern (data not shown). While these studies do not rule out the possibility that some of the epitopes were lipid in nature, they do diminish the possibility that the epitopes were simply adsorbed to the surface of the vacuole or were derived solely from fatty acids. If Triton X-100 was added prior to addition of the antiserum, then the confocal images revealed the presence of a bright, almost uniform disc (Fig. 10C). This image is consistent with the antibody gaining access to the internal contents of the inclusion membrane.

DISCUSSION

Chlamydia are internalized by endocytic mechanisms, and within 2 to 12 h, the infectious EB form of the bacteria transforms into the noninfectious replicating reticulate body form. As bacterial multiplication ensues, the inclusion expands. While it is clear that the membrane surrounding the newly internalized bacteria is initially derived from the host plasma membrane, the source of the membrane that comprises the maturing inclusion is unknown. Our studies demonstrate that the maturing chlamydial inclusion is not found within the endocytic pathway and that the expanding inclusion membrane is not derived from the plasma membrane by continued endocytic activity. These studies used content markers (HRP and lucifer yellow), membrane markers (Tf), and antibodies to membrane components (Tf receptors, mannose 6-phosphate receptors, and Lamps).

Studies on the pH of vesicles containing intracellular parasites such as *Legionella pneumophila* (17, 18) and *Toxoplasma gondii* (37) indicated that the luminal pH is much higher (0.8 pH units) than that of endocytic vesicles containing either bacteria or heat-killed parasites. While these intracellular pathogens may be capable of modifying the pH of the endosomal membrane, an alternate hypothesis suggested by this study is that the pathogens are internalized by areas of plasma membrane which are not part of the endocytic apparatus and are devoid of the ATP-hydrogen transport system. If such vesicles did not contain the hydrogen ATPase, they would not become acidic, and thus the vesicles might be biochemically different from endosomes. This possibility also leads to a hypothesis on how these organisms may avoid fusion with lysosomes. Rather then the intracellular parasite actively preventing fusion between newly internalized vesicles and the intracellular endocytic apparatus, the membrane surrounding the intracellular parasite may simply lack fusion or recognition signals on the cytosolic surface. That is, fusion of endosomal contents may be a property of specific membrane proteins which are internalized in early endocytic vesicles and are not within the inclusion. Increasing lines of evidence indicate that fusion events require targeting information and that the presence or absence of such targeting information determines whether vesicles will fuse and the specificity of that fusion (5, 39, 43). If the parasite induces the internalization of a plasma membrane vesicle which lacks fusion signals, then such vesicles may not fuse with lysosomes. Evidence supporting this hypothesis has been published by Joiner et al. (21), who demonstrated that transfection of fibroblasts with plasmids containing the cDNA for Fc receptors resulted in the expression of functional Fc receptors. These cells can internalize antibody-coated *T. gondii*, and vesicles containing the parasites fused with lysosomes (21). Vesicles containing antibody-coated *T. gondii* internalized by Fc receptors which lack cytoplasmic tails do not fuse with elements of the endocytic apparatus. Vesicles containing *T. gondii* internalized by Fc receptors which contain cytoplasmic tails do fuse with lysosomes (21). These results suggest that fusion requires targeting signals contained in the cytoplasmic tail of the Fc receptor. An equally tenable hypothesis is that the parasite-containing vesicles do contain classic endosomal components such as H^+-ATPases and lysosomal targeting molecules but that these molecules are rapidly removed by recycling processes and, once removed, are not replaced.

The fact that chlamydiae multiply within a membranous system distinct from the endocytic apparatus clearly rules out the hypothesis that the late inclusion membrane is derived from continued endocytic activity. If the inclusion membrane is recruited from the plasma membrane, the method of recruitment would require some novel, undescribed transport system. The fact that we did not observe any staining of the inclusion membrane with either anti-Tf receptor antibodies or three different lectins makes it unlikely that the inclusion membrane is composed of plasma membrane components.

The late chlamydial inclusion membrane could be increased by fusion with internal membranes. The surface area of internal membranes, such as the Golgi apparatus and ER, is large relative to the surface area of plasma membrane (11) and could conceivably serve as a reservoir of membrane. The proof of such fusion events would require the demonstration of internal membrane constituents or their contents as part of the inclusion. We used three different markers, two of which were viral constructs expressing the HA epitope. One of the HA epitopes was a content marker, and the other HA epitope was a membrane marker. The fact that neither marker was found associated with inclusions strongly suggests that these membranes do not fuse with the inclusion. Further support for this conclusion comes from examination of the distribution of the mannose 6-phosphate receptor. This molecule, while found on the plasma membrane and early endosome, is highly concentrated in the Golgi apparatus and late endosome. We did not observe any alternation in the distribution of this marker in infected cells. We did not observe any staining of the inclusion with the antibody against the mannose 6-phosphate receptor. Thus, we have confirmed the HA epitope observations with host cell proteins (i.e., mannose 6-phosphate receptor). Hackstadt et al. (12) have recently demonstrated that sphingomyelin endogenously synthesized from C_6-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-ceramide in the Golgi apparatus appears to be incorporated into the cell wall of the parasite, suggesting a connection between the Golgi apparatus and the chlamydial inclusion. However, host proteins from the Golgi apparatus and ER have not been identified within the inclusion membrane. Finally, by the use of an antibody against Lamp-1 and Lamp-2, we demonstrated no evidence of fusion with late endosomal or lysosomal components. This result extends the observation that chlamydial inclusions do not fuse with lysosomes either as they initially enter the cell or once they are within the cell.

Almost invariably, mature inclusions were observed immediately adjacent to the nucleus (Fig. 1, 4, 5, and 6). We do not think that this distribution reflects an active transport of inclusions. Rather, this distribution is the result of the large inclusion being displaced from the peripheral cytoplasm, perhaps as a result of a lack of interaction with the cytoskeletal network. Other large structures, such as sucrosomes (Fig. 1) (19) or large lysosomes seen in the Chediak-Higashi syndrome (Fig. 1) (29), show a similar distribution. The Golgi apparatus, much of the ER, and recycling endosomes show a similar distribution. Thus, the close approximation of these structures to the inclusion (Fig. 4 and 6) does not necessarily connote a functional relationship.

Studies by Matsumoto (27) show electron micrographs in

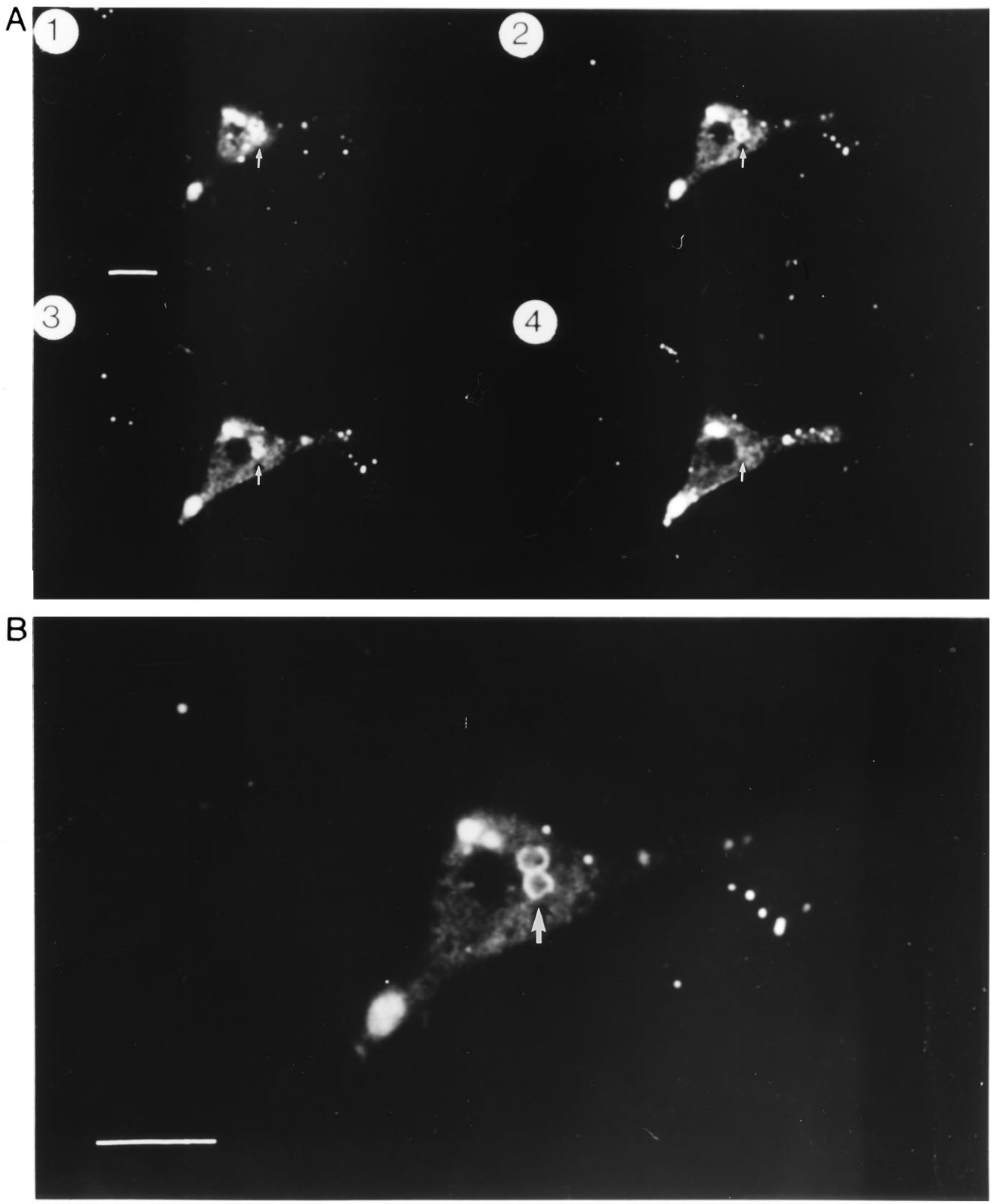

FIG. 10. Distribution of mouse anti-chlamydial membrane antibody in semiintact *Chlamydia*-infected cells. MDBK cells at 36 h after infection with *C. psittaci* were permeabilized by using either the "bouncing bead" procedure (A and B) or 0.2% Triton X-100 (C). Permeabilized cells were incubated with our mouse anti-chlamydial membrane antiserum followed by an FITC-labeled goat anti-mouse antibody. (A) Confocal series of an inclusion at 2.0-μm increments; (B) expanded image of panel A, section 2; (C) fluorescent micrograph captured by using Oncor Image software as described in Materials and Methods. Arrowheads mark corresponding inclusions. Bar, 10 μm.

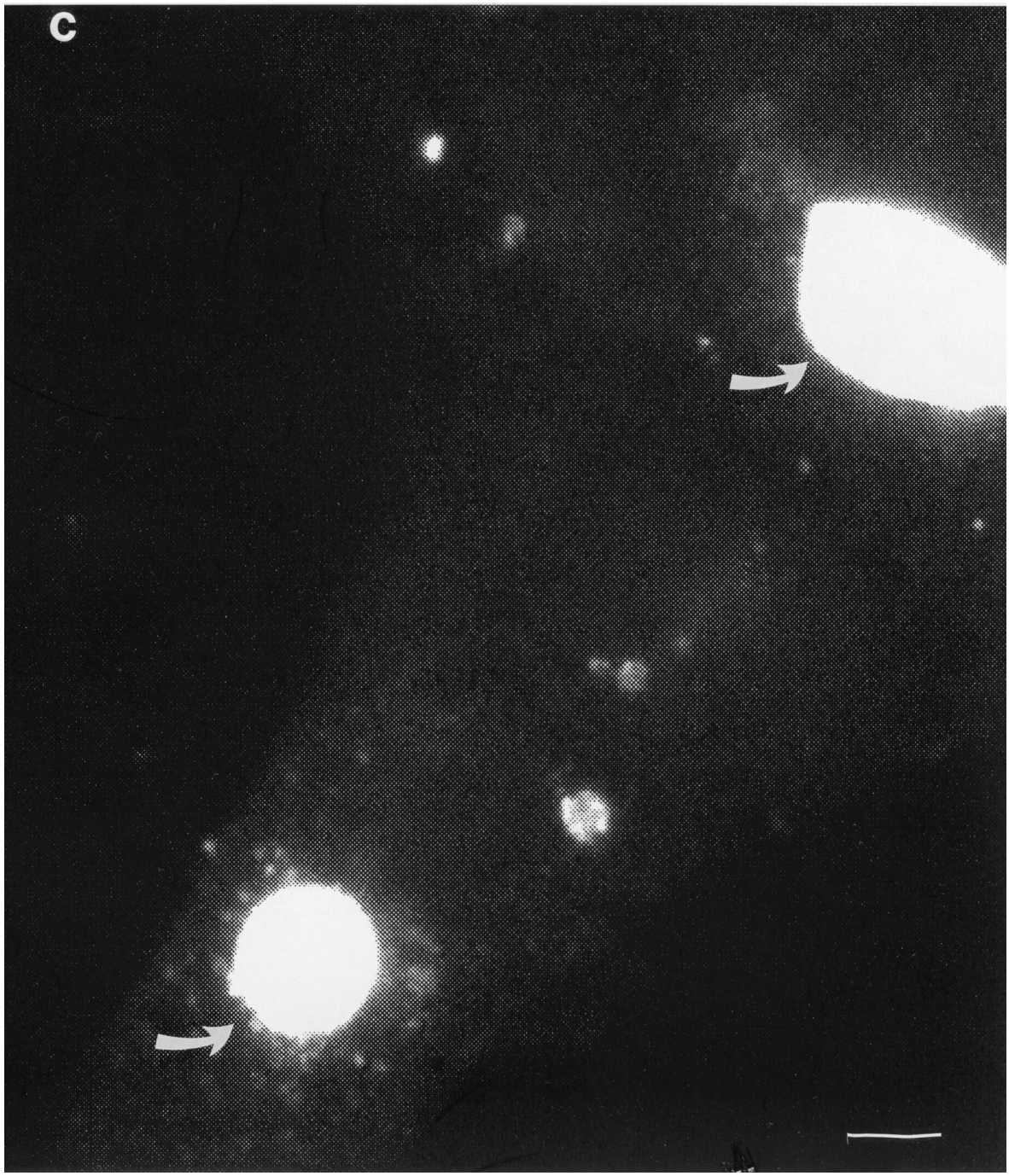

FIG. 10—*Continued.*

which chlamydial projections appear to penetrate the inclusion membrane. These structures are thought to be involved in transport of molecules, including ATP, from the cytosol to the bacteria within the inclusion. The presence of such structures suggest an intimate physical connection between the bacteria and the inclusion membrane. To test this possibility, we stained the exterior surface of the inclusion with antibodies directed against *Chlamydia* membrane. Selective permeabilization of cells allowed the antiserum access to the exterior (cytoplasmic) surface of the inclusion. The antiserum directed against membranes from *Chlamydia*-infected cells was capable of staining the surface of the inclusion membrane without staining the internal contents of the chlamydial inclusion. This result suggests that *Chlamydia*-specified components comprise, at least in part, the inclusion membrane.

These observations are consistent with those of Rockey et al. (31, 32), who observed that antibody from guinea pigs which had been infected with *C. psittaci* detected proteins specific to infected cells and not present in mature EB. Immunofluorescence of detergent-treated infected cells with this antiserum

demonstrated staining of the periphery of the inclusion, suggesting that the epitope was concentrated in or proximal to the inclusion membrane. We did not distinguish whether the antiserum used in our study was directed against both reticulate body- and EB-specific epitopes, although we think that this is probable. We think that we have demonstrated that most of the antiserum is directed against proteinaceous components. Particularly compelling are the observations that the mouse antiserum does not immunoprecipitate proteins labeled with [^{35}S]methionine prior to infection and that subsequent Triton X-100 extraction does not alter the immunofluorescent pattern of selectively permeabilized cells. Regardless of what developmental form the antiserum is directed against, it clearly recognizes epitopes present on the cytoplasmic surface of the inclusion.

The relative absence of host proteins and the ability to selectively stain the exterior surface of the inclusion with antichlamydial antiserum lead to the conclusion that the growing inclusion membrane is derived from *Chlamydia*-specified components. A variety of taxonomically unrelated intracellular parasites live within vesicles which do not appear to be part of the endocytic apparatus; these include *T. gondii*, legionellae, and mycobacteria. While in all instances the invading parasite is initially surrounded by host plasma membrane, little is known about the origin of the growing inclusion or parasitophorous vacuolar membrane. There is evidence that the vacuolar membrane which surrounds the intracellular protozoan parasites is derived from both the host and the parasite. The membrane surrounding the protozoans may be initially derived from the plasma membrane as a result of the entry of the protozoan into the host cell (20). Subsequently, this membrane is modified by parasite-secreted material. Many of the parasites, for example *T. gondii* and *Plasmodium falciparum*, have specific secretory organelles called rhoptries, which contribute vesicular material into the parasitophorous vacuole (3, 28, 41). This material has been isolated and is composed of both proteins and lipids (6, 36). Immunocytochemical and biochemical studies have demonstrated that some of these components become associated with and are part of the parasitophorous vacuolar membrane (3, 23, 35). Studies of *P. falciparum*-infected erythrocytes have demonstrated that antigens present within the parasite can also become part of the vacuolar membrane which surrounds the parasite (22). Morphological studies on a number of intracellular protozoan parasites indicate the presence of vesicular material within the parasitophorous vacuole that appears to be elaborated or shed from the parasite (40). Images can be found in which this material, which often has a myelin-like appearance, becomes contiguous with the vacuolar membrane (35, 36). These results lead to the conclusion that the vacuolar membrane of protozoan parasites, particularly after infection, are composed of parasite-derived material. We have also observed images which reveals what looks like an onion skin membrane derived from the bacteria which is contiguous with the inclusion membrane (data not shown). These images suggest an intimate association of the bacteria with the inclusion membrane as observed by Matsumoto (27).

Rockey et al. (31) have gone further to isolate a *C. psittaci* gene coding for a protein localized in the inclusion membrane of infected cells. However, this gene is not present in *C. trachomatis*. Our studies demonstrate several different-molecular-weight *Chlamydia*-specified proteins within the inclusion membrane. Together, these data suggest that more than one *Chlamydia*-specified protein may be found within the inclusion membrane and that membrane expansion is driven by fusion with bacterium-derived material. While it has been suggested that *L. pneumophila* may fuse with ER-derived vesicles (17), less information is available regarding the composition of the membrane surrounding other bacteria which live as intracellular parasites, such as mycobacteria. Further studies are required to determine if bacterial modification of host membranes may be a general mechanism by which inclusions gain membrane to accommodate the expanding bacterial cell mass.

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