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In Vitro Neutralization of *Chlamydia trachomatis* with Monoclonal Antibody to an Epitope on the Major Outer Membrane Protein

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A murine monoclonal antibody, which binds to an epitope on the major outer membrane protein of *Chlamydia trachomatis* and with species specificity in the micro-immunofluorescent assay, effectively neutralized in vitro two antigenically distinct serovars of *C. trachomatis*. Optimal concentrations of both organism and antibody were required to produce maximal neutralization of the organism. Neutralization was less effective and more variable at lower dilutions of antibody than at higher dilutions, suggesting a prozone phenomenon. A radiolabeled attachment assay demonstrated that attachment of elementary bodies was unaffected by earlier treatment with antibody and that neutralization occurred at a step after attachment. The epitope to which this antibody is directed, on the major outer membrane protein of *C. trachomatis*, may have an important role in determining infectivity of the organism.

Immune mechanisms are involved in regulating *Chlamydia trachomatis* infection. Both in vivo and in vitro observations suggest that antibody limits the extent of chlamydial infection. Secretory immunoglobulin A (IgA), in particular, has been found to inversely correlate with quantitative recovery of the organism from the human endocervix (5). In ocular infection of the owl monkey, secretions obtained from the eyes of children with trachoma were found to neutralize infection (11). In mice, passively transferred antibody conferred protection to challenge infection with the mouse pneumonitis strain of *C. trachomatis* (17).

Several different investigators have shown that antibody can neutralize *C. trachomatis* in vitro (1–3, 9, 13). In particularly revealing experiments, Caldwell and Perry (7) reported in vitro neutralization of *C. trachomatis* with rabbit polyclonal IgG raised against purified major outer membrane protein (MOMP) of the organism. We report that murine monoclonal antibody is also able to neutralize *C. trachomatis* under in vitro conditions. We have examined the kinetics for and stage at which neutralization occurs with this monoclonal antibody.

MATERIALS AND METHODS

Growth and purification of *C. trachomatis*. *C. trachomatis* strains L2/434/Bu (serovar L_2) and UW12/UR (serovar I) were used (courtesy of C.-C. Kuo, University of Washington, Seattle). Organisms were grown in HeLa 229 cell monolayers in 175-cm^2 polystyrene culture flasks (Nuclon; GIBCO) containing 50 ml of Eagle minimal essential medium (MEM), 10% fetal calf serum, 2 mM L-glutamine, 25 μg of gentamicin per ml, 100 μg of vancomycin per ml, 25 U of nystatin per ml, and 0.5 μg of cycloheximide per ml (MEM-10 with cycloheximide). Serovar I was grown in HeLa cells pretreated with DEAE-dextran (30 μg/ml for 20 min before infection). At 48 to 72 h postinfection when ≥90% of cells contained inclusions, the culture medium in each flask was poured off, and cells were harvested with 4-mm glass beads in 10 ml of cold Hanks balanced salt solution. Cells were ruptured by sonication for 35 s with a probe intensity of 35 to 40. HeLa cell debris was removed by centrifugation at 500 × *g* for 15 min at 4°C. *C. trachomatis* was concentrated from the supernatant by centrifugation at 30,000 × *g* for 30 min. The pellet was resuspended in 0.35 M sucrose–10 mM sodium phosphate–5 mM L-glutamine acid (SPG; pH 7.2) at a concentration of 5 ml/flask. Organisms thus prepared were frozen at −70°C until needed in individual assays. Quantification of infectious units was performed by the inclusion counting method of Furness et al. (8). Inclusions were counted in 30 fields at ×400 magnification with the aid of a micrometer and expressed as the number of inclusion-forming units (IFUs) per cover slip.

Whole purified elementary bodies (EBs) of L_2 were treated with Sarkosyl exactly as described by Caldwell et al. (6), and both solubilized proteins and the insoluble pellet containing the chlamydial outer membrane were saved.

SDS-polyacrylamide gel electrophoresis. Supernatant and pellet obtained after Sarkosyl treatment of whole EBs of L_2 were solubilized in 0.1 M Tris (pH 6.8)–2.5% sodium dodecyl sulfate (SDS)–5% 2-mercaptoethanol–20% glycerol–0.001% bromphenol blue. Ca. 5 μg of protein were electrophoresed through 12.5% polyacrylamide gels 0.75 mm in thickness. The gels were stained with a highly sensitive silver stain to visualize separated polypeptides (10).

Monoclonal antibody. A murine monoclonal antibody, designated AE11, was provided by C.-C. Kuo. The method of production and characterization of similar monoclonal antibodies has been previously described (14). The monoclonal antibody was provided, both as a culture supernatant and as ascitic fluid. The isotype of this monoclonal was IgG3. The culture supernatant containing the monoclonal antibody reacted against all serovars of *C. trachomatis* in the micro-immunofluorescent assay (16).

Immunoblot assay. To determine the polypeptide specificity of this antibody, an immunoblot assay was performed. Sarkosyl-solubilized, purified EBs of *C. trachomatis* L_2 serovar were electrophoresed on 12.5% acrylamide gels for 6 h. The electrotransfer procedure was a modification of the method of Towbin et al. (15) in that electrophoresis from the gel to the nitrocellular paper proceeded for 16 h. Culture supernatant monoclonal antibody was added at a dilution of 1:250. Bound antibody was detected by the addition of anti-

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mouse IgG (Cappel Laboratories, Cochranville, Pa.) which was peroxidase labeled. The peroxidase substrates diaminobenzidine and H_2O_2 were added, and a brown reaction product was visible where antibody had bound.

Purification of monoclonal IgG from mouse ascites fluid. Purified IgG was prepared from mouse ascites fluid with a protein A-Sepharose CL-4B column (Pharmacia, Uppsala, Sweden). One milliliter of mouse ascites containing monoclonal AE11 was passed through a 0.45-μm Millipore filter. This was then passed through a column (1 by 20 mm) of 1.5 g of protein A-Sepharose CL-4B previously equilibrated with 0.05 M Tris–0.15 M NaCl–0.02% sodium azide (pH 8.4). The column was washed with 15 volumes of Tris buffer, 2-ml fractions were collected, and the optical density at 280 nm was followed. When no further protein was eluted from the column, bound IgG was eluted by adding 0.1 M sodium acetate (pH 4.0). Two-milliliter fractions were collected into an equal volume of 0.5 M phosphate buffer (pH 7.5). The optical density at 280 nm was determined, and fractions were pooled and dialyzed against three changes of 0.01 M phosphate-buffered saline (PBS). The protein concentration of the purified IgG was determined by the dye-binding method (4).

Neutralization assay. Equal volumes (200 μl) of antigen and antibody or PBS control at appropriate dilutions were mixed and incubated at 37°C for 30 min in a shaker water bath. No complement was used, and assays were done in triplicate. Residual chlamydial infectivity was assayed by inoculating 100 μl of the incubated antigen-antibody mixture onto 24-hour-old cover slip cultures of HeLa 229 cells (seeded at 4×10^5 cells per cover slip). The inoculum was allowed to adsorb at room temperature for 2 h without centrifugation. The monolayers were then washed with Hanks balanced salt solution and refed with MEM-10 with cycloheximide. The cover slip cultures were incubated at 35 to 37°C for 48 to 72 h, fixed with absolute methanol, and stained for chlamydial inclusions with monoclonal antibody AE11 and fluorescein-conjugated anti-mouse immunoglobulin (Cappel Laboratories).

Attachment assay. Radiolabeling of the organisms and attachment of ^{14}C-labeled EBs to HeLa cell monolayers were carried out as previously described (7). Briefly, ^{14}C-labeled EBs were grown and harvested as outlined above, except that after the initial 10 h of growth, complete MEM-10 was substituted with MEM-10 containing 1/10 the normal concentrations of amino acid, 1 μCi of ^{14}C-amino acids (L-amino acid mixture [specific activity, 1.78 mCi/mg; ICN Pharmaceuticals, Inc.]), and 1 μg of emetine per ml. The ^{14}C-labeled EBs were purified by pelleting through 35% Renografin in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)–0.15 M NaCl (HEPES buffer) and banded in discontinuous 40 to 52% Renografin density gradients. The band was washed twice with HEPES buffer and suspended in SPG.

To assay for attachment of uncoated and antibody-coated L_2 EBs to HeLa cells, ca. 10^6 IFUs of ^{14}C-labeled EBs were incubated with either PBS or monoclonal antibody at 1:102,000 dilution at 37°C for 30 min. This dilution of monoclonal antibody was chosen because previous experiments showed it to produce optimal neutralization. One hundred microliters of this reaction mixture was inoculated into each of three cover slips containing ca. 10^5 24-hour-old HeLa 229 cells. The inoculum was allowed to adsorb for 2, 15, 30, 45, 60, and 90 min. After adsorption, the inoculum was removed, and the monolayer was washed three times with Hanks balanced salt solution. The three monolayers were lysed with 200 μl of 2% SDS–distilled water (wt/vol) at selected time intervals. Ten microliters of each of the cell lysates were diluted in 90 μl of water and transferred to glass scintillation vials to which 10 ml of Aquasol (New England Nuclear Corp., Boston, Mass.) was added and counted in a liquid scintillation counter (1215 Rackbeta II; LKB Instruments, Inc., Rockville, Md.).

RESULTS

Monoclonal antibody binds to an epitope on MOMP of *C. trachomatis*. The SDS-polyacrylamide gel electrophoresis profile of Sarkosyl-treated EBs from L_2 is shown in Fig. 1. Five micrograms of protein of the Sarkosyl supernatant was applied to lane A, and 5 μg of the Sarkosyl pellet was applied to lane B. The MOMP of L_2 is ca. 39,000 daltons and strongly enriched in the Sarkosyl-insoluble pellet. The immunoblot with monoclonal antibody AE11 after electrotransfer with both of these preparations is shown in lanes C and D. The antibody detects an epitope found only on MOMP in lane D.

Neutralization of two different serovars of *C. trachomatis*. The epitope on MOMP detected by the monoclonal antibody is surface exposed and common to all serovars of *C. trachomatis*, as determined in the micro-immunofluorescent assay. It therefore was of interest to determine whether AE11 could neutralize different serovars of *C. trachomatis*. This monoclonal antibody was able to effectively neutralize two antigenically distinct serovars of *C. trachomatis*, L_2 and I (Table 1).

This result suggests that antibody to a species-specific epitope can neutralize multiple serovars of *C. trachomatis*. In addition, neutralization appeared to be less effective at the highest concentrations of organisms tested. Therefore, in subsequent experiments, concentrations of organisms producing between 1,000 and 10,000 inclusions per cover slip were used.

The relative neutralization titers of serovars L_2 and I were then compared. Under the conditions of the assay, neutralization of I was ca. 60-fold more effective than that of L_2 (see

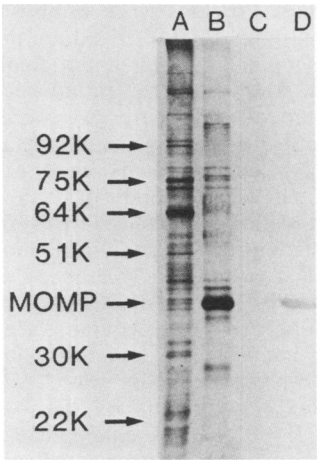

FIG. 1. SDS-polyacrylamide gel electrophoresis and immunoblot of *C. trachomatis* serovar L_2 treated with Sarkosyl as described in the text. Lane A, Sarkosyl supernatant; lane B, Sarkosyl pellet stained with silver after electrophoresis through a 12.5% polyacrylamide gel. MOMP is ca. 39,000. Lanes C and D, Electrotransfer of lanes A and B, respectively. These lanes were reacted with monoclonal antibody AE11 and probed with peroxidase-labeled anti-mouse IgG. The monoclonal antibody detects an epitope found on MOMP in lane D.

TABLE 1. Neutralization of *C. trachomatis* serovars L_2 and I with murine monoclonal antibody AE11 diluted 1:10 in PBS

C. trachomatis serovar	No. of IFU/cover slip		% Reduction
	PBS-treated organism	Antibody-treated organism	
L_2	4.86×10^4	8.36×10^2	98
	1.23×10^4	0	100
	2.36×10^3	0	100
I	3.2×10^4	4.34×10^4	0
	2.0×10^3	0	100
	3.04×10^2	0	100

Fig. 2). The 50% neutralizing endpoints, as calculated by the method of Reed and Muench (12), for serovars I and L_2 were 1:480,000 and 1:8,000, respectively. In addition, at lower dilutions of monoclonal antibody, neutralization of L_2 was less effective than at higher dilutions.

Neutralization of chlamydiae with purified monoclonal antibody. Experiments thus far described used crude culture supernatants as the source of monoclonal antibody. To precisely define the role of antibody in the neutralization, ascitic fluid collected from mice previously inoculated with AE11 hybridoma was passed through a protein A-Sepharose CL-4B column, and purified IgG antibody was collected. This antibody was adjusted to a protein concentration of 156 μg/ml, and serial fourfold dilutions were prepared. Purified monoclonal IgG neutralized *C. trachomatis* to a higher titer than did crude culture supernatant of antibody (Fig. 3). The 50% neutralizing endpoint for serovar L_2 with purified IgG was 1:403,645 and 1:309,030 in two separate experiments. The neutralization produced with culture supernatant antibody is shown for comparison, and the 50% neutralizing endpoint was 1:8,000. Again, with both preparations of antibody, neutralization was less effective and more variable at lower dilutions of antibody than at higher dilutions. This observation suggested a prozone phenomenon.

Effect of monoclonal antibody on attachment of *C. trachomatis* to HeLa cells. Previous work established that rabbit polyclonal IgG antibody to MOMP did not produce neutralization of *C. trachomatis* through inhibition of attachment to the host cell (7). Rather, neutralization occurred at some step after attachment. Therefore, we were interested in determining whether monoclonal antibody to AE11 acted similarly. Radiolabeled organisms were prepared as described and treated with antibody or PBS. After 30 min, pretreated chlamydiae were added to fresh HeLa cell monolayers. At selected intervals, individual monolayers were extensively washed to remove unattached organisms, and cell monolayers were harvested. Monolayers were treated with SDS, and radioactivity was counted (see Fig. 4). At the end of 90 min of incubation, the cell-associated counts per minute with PBS-pretreated EBs was 1,670 ± 320; with monoclonal antibody-pretreated EBs, the counts per minute was 1,400 ± 265 ($P > 0.1$). Thus, chlamydiae pretreated with monoclonal antibody or PBS associated with HeLa cells equally, rapidly, and to the same extent. We concluded from this experiment that this antibody does not inhibit attachment.

DISCUSSION

Although *C. trachomatis* infections are frequently persistent, in vivo data suggest that antibody has an important modulating influence on the extent of infection (5). What structures on the organism and which steps in the life cycle are susceptible to inhibition are at present unclear. Several investigators have shown that whole sera are effective in neutralizing the homologous strain of chlamydiae; however, neutralization titers have generally been low, and at times incomplete neutralization has occurred (1–3, 9, 13). Collectively, the results have suggested that neutralization is type specific. Howard (9) noted that neutralization titers could be substantially increased by the addition of complement. The relevance of this observation to natural infection, however, is uncertain since most chlamydial infections are limited to a mucosal surface and occur in a complement-deficient environment. The antigenic basis and mechanism for neutralization have not been defined in any of these studies.

Caldwell et al. (7) were the first to demonstrate that a

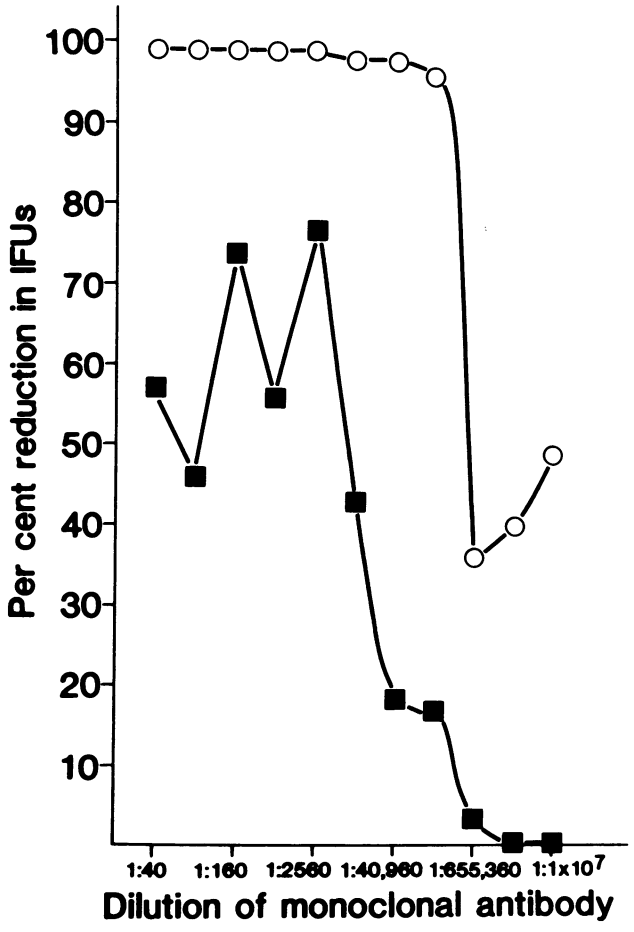

FIG. 2. Titration of reduction in infectivity of *C. trachomatis* serovars L_2 (■) and I (○) with fourfold dilutions of murine monoclonal antibody AE11 hybridoma culture supernatant. EBs from serovars L_2 and I were preincubated with either PBS or the monoclonal antibody at various dilutions for 30 min at 37°C. The resulting antigen-antibody mixtures were assayed for residual infectivity by inoculation onto 24-hour-old HeLa 229 cell monolayers grown on cover slips. IFUs in these cell monolayers were quantitated by an immunofluorescent method after incubation at 37°C for 48 to 72 h. The results are expressed as percentages of reduction in IFUs at each antibody dilution as compared with the IFUs for the PBS control.

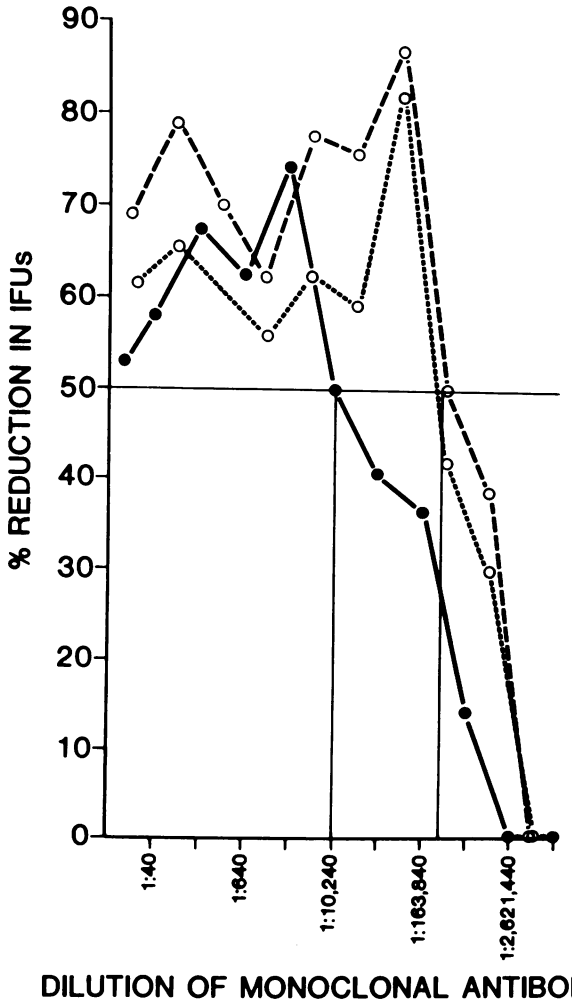

FIG. 3. Titration of reduction in infectivity of *C. trachomatis* serovar L_2 with fourfold dilutions of murine monoclonal antibody AE11 culture supernatant (●) and purified IgG from ascitic fluid (○). L_2 EBs were preincubated at 37°C for 30 min with PBS or fourfold dilutions of monoclonal antibody. Residual infectivity in the resulting mixture was assayed by inoculating it onto 24-hour-old HeLa 229 cell monolayers grown on cover slips. After incubation at 37°C for 48 h the cells were fixed with methanol, and chlamydial inclusions in these cells were stained and counted by an immunofluorescent method. The results are expressed as percentages of reduction in IFUs for each antibody dilution as compared with the number of IFUs for the PBS control.

purified antigenic component of the organism, MOMP, could induce antibody which was able to neutralize the homologous strain in vitro. A series of experiments by these investigators disclosed that polyclonal IgG antibody to MOMP could produce neutralization both with and without added complement. Complement-independent neutralization did not depend on aggregation of the organism, inhibition of attachment, or entry into the host cell. Rather, neutralization occurred at a step after endocytosis. Neutralization was dependent on intact, dimeric IgG molecules, since papain digests of these antibodies were ineffective. Based on these results, the authors speculated that intact IgG on the surface of the organism could promote phagolysosomal fusion, inhibit essential nutritional needs of the organism, or prevent differentiation of EBs into reticulate bodies and thereby account for inhibition of inclusion formation. These investigations did not determine whether polyclonal antibody to L_2 MOMP could effectively neutralize other serovars of *C. trachomatis*.

Stimulated by these results, we were interested in determining whether a monoclonal antibody of predefined specificity reacting with epitopes on MOMP of all known serovars of *C. trachomatis* could also produce in vitro neutralization. Our results demonstrate that such antibody reliably neutralizes two antigenically distinctive serovars, I and L_2, of *C. trachomatis*, thus demonstrating that neutralization can occur with an antibody to a species-specific epitope on MOMP of *C. trachomatis*.

Optimal concentrations of both organism and antibody were required to produce maximal neutralization. In particular, inocula producing between 1,000 and 10,000 IFUs per cover slip in control cultures were required to reliably show neutralization. At higher concentrations of organism, neutralization was very much less effective. In addition, at high concentrations of antibody, neutralization was also less effective. This prozone phenomenon was unexpected and is somewhat difficult to explain but could relate to the mechanism by which neutralization occurs. For instance, if neutralization occurs through a postentry mechanism (such as cross linking of MOMP molecules on the surface of EBs, thereby preventing differentiation to the reticulate body), it may be that with high antibody concentrations more univalent interaction and less cross linking of different MOMP molecules occur.

Studies with purified IgG monoclonal antibody to MOMP demonstrated that this antibody produced neutralization through a mechanism other than inhibition of attachment. Both antibody- and PBS-pretreated chlamydiae had equally rapid cell association kinetics. Preliminary results with elec-

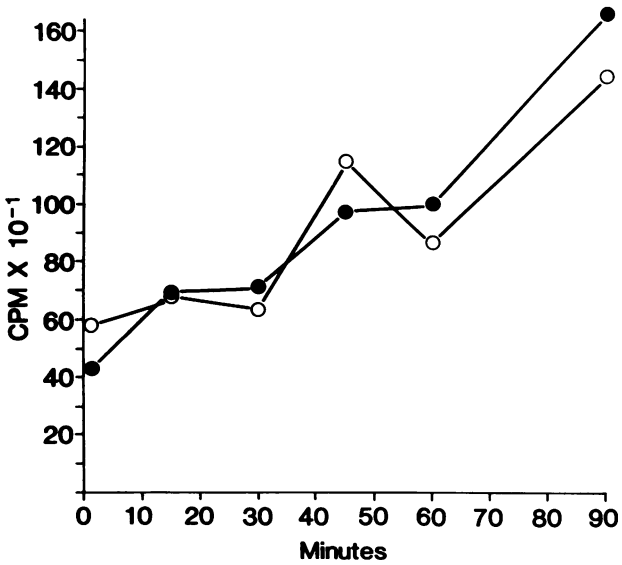

FIG. 4. Effect of murine monoclonal antibody AE11 on the attachment of ^{14}C-labeled L_2 EBs to HeLa 229 cells. Symbols: ○, L_2 EBs preincubated with purified monoclonal IgG; ●, L_2 EBs preincubated with PBS. ^{14}C-amino acid radiolabeled EBs of serovar L_2 were pretreated with either purified monoclonal IgG or PBS for 30 min at 37°C and then inoculated into 24-hour-old HeLa 229 cell monolayers. At selected time intervals, the monolayers were washed to remove unattached EBs, lysed with 2% SDS in distilled water, and assayed for radioactivity.

tron microscopic studies with ferritin-conjugated anti-mouse IgG in an immune electron microscopy technique further showed that attachment of antibody- and PBS-pretreated chlamydiae was rapidly followed by endocytosis (data not shown). Our results thus confirm those of Caldwell et al. (7) that neutralization of chlamydiae with an antibody to MOMP occurs postattachment. Further electron microscopic work will be required to discern whether neutralization is the result of phagolysosomal fusion or prevention of intracellular differentiation.

The epitope recognized by this monoclonal antibody is of particular interest since antibody with this particular specificity in vivo may confer resistance to multiple serovars of *C. trachomatis*. Identification and biosynthesis of the peptide sequence of this epitope could allow for its testing as a component in a candidate chlamydial vaccine.

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