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In Vitro Neutralization of *Chlamydia trachomatis* by Monovalent Fab Antibody Specific to the Major Outer Membrane Protein

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Monovalent Fab antibodies to serovar- and subspecies-specific epitopes of the major outer membrane protein (MOMP) of *Chlamydia trachomatis* neutralized infectivity for hamster kidney cells by preventing chlamydial attachment. These findings exclude the aggregation of chlamydiae as a mechanism of anti-MOMP neutralization and provide additional evidence in support of the MOMP as a chlamydial adhesin.

We previously described the in vitro neutralization of *Chlamydia trachomatis* infectivity for Syrian hamster kidney (HaK) cells with monoclonal antibodies (MAbs) specific to epitopic determinants located in surface-exposed variable domains (VDs) of the major outer membrane protein (MOMP) (7). These MAbs neutralized infectivity by preventing chlamydial attachment. A potential shortcoming of those studies, however, was that bivalent immunoglobulin G's (IgGs) were used in the neutralization assays, and therefore aggregation could not be excluded as a possible neutralization mechanism. To more thoroughly define the mechanism by which MOMP-specific antibody neutralizes *C. trachomatis* infectivity, we prepared monovalent Fab antibody fragments from two neutralizing MAbs. We show here that monovalent Fab antibody to MOMP neutralizes *C. trachomatis* infectivity in vitro by preventing chlamydial attachment. These findings exclude aggregation as the mechanism of neutralization and provide further support for a role of the MOMP in chlamydial attachment.

C. trachomatis serovars A (strain Har-13) and B (strain TW-5/OT) were grown and radiolabeled with ^{14}C-amino acids in HeLa 229 cells as previously described (3). Elementary bodies (EBs) were isolated by centrifugation in discontinuous gradients of Renografin-76 (diatrizoate meglumine-diatrizoate sodium; Squibb Diagnostics, New Brunswick, N.J.) (4). The production, purification, and characterization of MAbs A-20 and DIII-A3 have been reported in detail elsewhere (8, 9). MAb A-20 is specific to serovar A MOMP and recognizes a surface-exposed contiguous epitope that maps to ^{70}DVAGLEKDPVA^{80} in VD I. MAb DIII-A3 has been determined to be species specific by Western blotting (immunoblotting) and recognizes a contiguous epitope contained within the sequence ^{293}FDVTTLNPTIAGAGDVK^{309} in VD IV (1). The DIII-A3 epitope is surface exposed on EBs of B and intermediate-complex serovars but is not accessible to antibodies on C-complex serovars (9). MAb L2I-45 is an L2 MOMP-specific antibody (2) and was used as the irrelevant control antibody in both neutralization and attachment assays. The L2I-45 MAb was standardized to contain the same protein concentration as the experimental MAbs A-20 and DIII-A3. Monovalent Fab fragments of protein A-purified MAbs were prepared by papain digestion with an ImmunoPure Fab preparation kit (Pierce, Rockford, Ill.) according to the instructions provided by the manufacturer. Purity and completeness of digestion were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 12.5% polyacrylamide gels under reducing and nonreducing conditions.

Neutralization assays were done by preparing serial twofold dilutions of test IgGs, irrelevant control IgG, or monovalent Fab antibodies in SPG (0.25 M sucrose–10 mM sodium phosphate–5 mM L-glutamic acid, pH 7.2). A 0.5-ml volume of EBs (4×10^5 IFU/ml for serovar A and 1.8×10^6 IFU/ml for serovar B) was mixed with an equal volume of antibody dilutions and incubated at 37°C for 1 h. A 200-μl volume of the mixtures was then inoculated in triplicate onto monolayers of HaK cells (4×10^5 cells per well) grown in 24-well plates (Linbro, Flow Laboratories, McLean, Va.) and incubated at 37°C for 2 h. The inoculum was removed, and the monolayers were washed with 500 μl of Hanks balanced salt solution (HBSS). The cells were fed with 1 ml of minimal essential medium containing 10% fetal calf serum and 0.5 μg of cycloheximide per ml and incubated for 48 to 72 h in 5% CO_2. The monolayers were washed twice with HBSS and fixed in absolute methanol for 15 to 20 min. Chlamydial inclusions were detected by indirect fluorescent-antibody staining (6) and quantified by counting 10 intermediate-magnification (160×) fields using an inverted epifluorescence phase-contrast microscope.

For attachment assays, ^{14}C-amino acid-labeled EBs (5.8×10^6 IFU [2.2×10^5 cpm]/ml for serovar A and 1.4×10^7 IFU [1.4×10^5 cpm]/ml for serovar B) were diluted in SPG containing antibody as described above and incubated at 37°C for 1 h. Monolayers of HaK cells grown in 24-well plates were washed with cold HBSS and placed on ice for 30 min. The HBSS was removed, and 200 μl of the ^{14}C-labeled EB-antibody mixtures was added to monolayers in duplicate. The plates were incubated at 4°C for 2 h, the inoculum was removed, and the cells were washed three times with cold HBSS. The cells were removed from the wells by the addition of 200 μl of 0.1 N NaOH followed by incubation at 70°C for 1 h. Each well was washed twice with 200 μl of NaOH, and the washes were pooled with the original lysates. Lysates were mixed with 10 ml of scintillation solution (Ready Value; Beckman Instruments, Inc., Fullerton, Calif.), and the radioactivity was determined by using a scintillation counter (LS 9000; Beckman Instruments).

The percent reduction in infectivity or attachment was calculated as follows: percent reduction = {[(IFU/cpm of control antibody) − (IFU/cpm of test antibody)]/(IFU/cpm of control antibody)} × 100.

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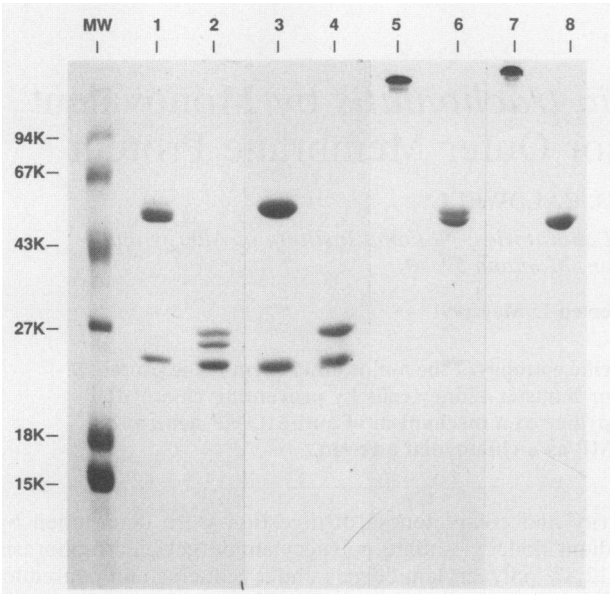

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of IgGs and isolated Fab fragments. Lanes 1 and 2 (MAb A-20 IgG and Fab) and 3 and 4 (DIII-A3 IgG and Fab) were solubilized in the presence of 2-mercaptoethanol. Lanes 5 and 6 (MAb A-20 IgG and Fab) and 7 and 8 (MAb DIII-A3 IgG and Fab) were solubilized in the absence of 2-mercaptoethanol. Characteristic electrophoretic mobilities were observed for purified Fab fragments when they were analyzed under reducing and nonreducing conditions.

MAb A-20 and DIII-A3 IgGs and their purified papain-derived proteolytic fragments were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing and nonreducing conditions (Fig. 1). The electrophoretic mobilities of both unreduced and reduced papain-digested MAb preparations were characteristic of Fab fragments. Under nonreducing conditions, the fragments migrated as 50-kDa polypeptides, and under reducing conditions, the fragments migrated as two distinct polypeptides at approximately 25 kDa. To determine that the Fab fragments of MAbs A-20 and DIII-A3 retained their original antibody-binding activity and specificity, they were iodinated (5, 7) and shown to react with intact EBs by dot-immunoblotting and with denatured MOMP by Western blotting (data not shown).

The neutralizing properties of intact MAb A-20 and DIII-A3 IgGs and their monovalent Fab fragments are shown in Fig. 2A. Both the bivalent IgGs and their corresponding Fab fragments neutralized chlamydial infectivity in a dose-dependent manner. MAb A-20 and MAb A-20 Fab fragments showed similar neutralizing efficiencies against serovar A at equivalent protein concentrations. Both antibodies reduced serovar A infectivity by 50% at a protein concentration between 3.12 and 1.56 μg/ml. At equivalent protein concentrations, MAb DIII-A3 IgG was more efficient in neutralizing serovar B infectivity than were its monovalent Fab fragments. A concentration between 3.12 and 1.56 μg of bivalent DIII-A3 IgG per ml was required to reduce serovar B infectivity by 50%, whereas 6.25 μg of Fab antibody per ml was required to achieve the same degree of neutralization. Heterotypic neutralization was not observed with either intact IgGs or their monovalent Fab fragments (data not shown). At equivalent protein concentrations, Fab preparations contain approximately 1.5 times the number of

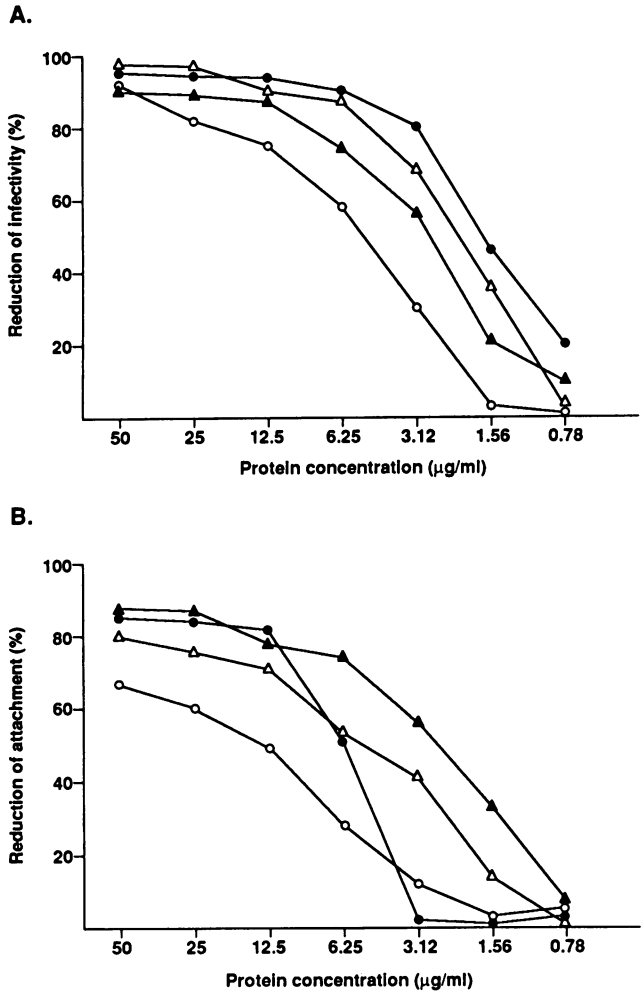

FIG. 2. Neutralization of infectivity (A) and inhibition of chlamydial attachment (B) by bivalent IgG and monovalent Fab antibody fragments specific to MOMP. ●, DIII-A3 IgG; ○, DIII-A3 Fab fragments; ▲, A-20 IgG; △, A-20 Fab fragments. *C. trachomatis* serovar B was used in assays with MAb DIII-A3 IgG and its Fab fragments. *C. trachomatis* serovar A was used in assays with MAb A-20 IgG and its Fab fragments. Percent reductions in infectivity and attachment were calculated as described in the text.

antibody-binding sites that bivalent IgG preparations do. Thus, the IgGs were more efficient neutralizing antibodies than their monovalent Fab fragments. This difference in neutralizing capability may be due to differences in the relative affinities of intact IgG and Fab fragments.

Attachment assays using ^{14}C-amino acid-labeled EBs were used to delineate the mechanism by which monovalent antibodies neutralized chlamydial infectivity. The assays were done at 4°C, which allows attachment but not internalization of EBs. Monovalent Fab antibodies and bivalent IgGs significantly inhibited the attachment of intrinsically labeled EBs to HaK cells (Fig. 2B). The percent reduction in the attachment of EBs to HaK cells at different antibody protein concentrations closely paralleled the results observed in the infectivity assays. MAb A-20 and its Fab fragments inhibited the binding of serovar A by about 50% at a protein concentration of 3.12 μg/ml. A protein concentration of 6.25 μg of MAb DIII-A3 IgG per ml inhibited serovar

B binding to HaK cells by 50%. MAb DIII-A3 Fab fragments were less efficient than bivalent IgG in inhibiting the attachment of serovar B to HaK cells, requiring 12.5 μg of DIII-A3 Fab antibody per ml to reduce the attachment of serovar B to HaK cells by 50%. Therefore, monovalent antibody specific to MOMP prevents chlamydial binding to host cells and excludes aggregation of EBs as a mechanism of chlamydial neutralization.

In our previous work describing in vitro neutralization of chlamydial infectivity, we used MAbs B-B6 and B-B5, which are specific to contiguous epitopes located in serovar B MOMP VD II and VD IV, respectively (7). For comparative reasons, we would have preferred to use the same antibodies in this study. However, Fab fragments prepared from both of these MAbs had significantly reduced binding affinities to MOMP compared with their bivalent IgGs. This was also apparent when we prepared Fab fragments from a number of well-characterized MAbs that we have produced in this laboratory. Fab fragments prepared from MAbs A-20 and DIII-A3 maintained excellent binding affinities to MOMP, as determined by their ability to react with intact EBs in the dot-immunoblot assay. For these reasons we were obligated to limit the scope of the results described here to these two MAbs. Nevertheless, our findings demonstrate that monovalent antibodies specific to contiguous epitopes located in MOMP VD I and VD IV neutralize infectivity by inhibiting chlamydial attachment. It is not clear, however, how antibody binding to MOMP VDs functions in preventing chlamydial attachment. We have suggested that the surface-exposed VDs of the MOMP contribute electronegative properties to the chlamydial surface that promote low-affinity electrostatic binding to host cells (7). Thus, antibodies may function directly by occupying these regions and preventing them from promoting electrostatic interactions with the host cell surface. Alternatively, antibodies binding to exposed MOMP VDs may function by steric inhibition, preventing other regions of the MOMP or other outer membrane components from interacting with a host cell receptor(s). Regardless of the mechanism, the ability of antibodies against contiguous epitopes located within MOMP VDs to prevent chlamydial attachment to eukaryotic cells provides a rational argument for the selection of epitopic determinants contained within these domains as vaccine targets for the production of chlamydia-neutralizing antibodies.

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