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Chlamydia trachomatis Major Outer Membrane Protein Variants Escape Neutralization by Both Monoclonal Antibodies and Human Immune Sera

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We have identified two families of novel *Chlamydia trachomatis* isolates with amino acid changes within the major outer membrane protein (MOMP) variable domains: one family of Da, D*, and D⁻ and one family of Ia and I⁻. In order to determine whether these MOMP variants can escape antibody neutralization of infectivity, we tested both the D and I prototype strains and the variants in a complement-independent in vitro neutralization assay. We found that variants can indeed escape neutralization by both monoclonal antibodies and polyclonal human immune sera that neutralize the prototype strain.

Given that natural immunity to *Chlamydia* infections is serovar specific (9), that the major outer membrane protein (MOMP) distinguishes the different *Chlamydia* serovars (1), and that monoclonal antibodies (MAbs) which recognize MOMP protect against infection in vitro and in vivo (17), MOMP has been considered a prime candidate for a vaccinogen. However, the exposed portions of MOMP have been shown to vary in sequence from one serovar to another (16). Further, we and others have identified new *Chlamydia trachomatis* variants with novel amino acid changes in MOMP variable domains (VDs) (3, 5, 8, 15).

There is currently little experimental data to indicate how much nucleotide variation in the MOMP VDs is sufficient for escape from antibody-mediated neutralization. It has been shown that serovar-specific MAbs neutralize only the serovar they specifically recognize (9), but there are many nucleotide differences between the MOMP VDs of different serovars. In the simplest case, can a variant with a single amino acid change in one of the MOMP VDs escape antibody-mediated neutralization? In the experiments reported here, we determined whether such variants and their parent strains were equally neutralized by MAbs and human polyclonal immune sera, using a complement-independent neutralization assay. We found that these variants do indeed escape both MAb and polyclonal human immune sera neutralizations while their parent strains are neutralized.

Two standard *C. trachomatis* laboratory strains, serovars D (UW-3/Cx) and I (UW-12/Ur), were used in this study. In addition, several serovariants, including Da (strains MT199 and TW448), D^-, D*, Ia (strains MT165 and NL1540), and I^-, all of which had been previously isolated and serotyped in our laboratory, were examined. We had previously determined the MOMP VD sequences of all these *Chlamydia* strains (8).

The microtiter plate inclusion typing method (13) was used to determine the reactivities of MAbs DP-1 and PE-5 which both recognize MOMP (11).

Sera were obtained from subjects with recently acquired *Chlamydia* infections, as confirmed by cervical, rectal, and urethral cultures. For this study, 10 patients whose sera had titers of anti-*Chlamydia* antibodies from 1:128 to 1:2,048 were selected. Antichlamydial antibodies in patient sera were determined by the microimmunofluorescence (MIF) method (14).

The in vitro neutralization assay developed by Su et al. (12) and Byrne et al. (4) was used in this study. Briefly, each *C. trachomatis* serovar or variant was diluted in SPG buffer (6) and added to the same volume of test MAb or human serum to a final concentration of 5×10^5 inclusion-forming units/ml. MAbs were diluted in SPG from 100 to 0.18 μg/ml and sera were diluted from 1,600 to 0.39 μg/ml before being added to the organisms. The *C. trachomatis*-antibody mixture was incubated with shaking for 1 h at 37°C, and an aliquot then added to each of two Syrian hamster kidney (HaK cells, obtained from Harlan Caldwell, Rocky Mountain Laboratories, Hamilton, Mont.) confluent monolayers in 24-well tissue culture plates. The inoculum was incubated statically at 37°C for 2 h and then removed, and the monolayers were rinsed with Hanks balanced salt solution. Medium (Eagle's minimal essential medium with 10% fetal calf serum and 1 μg of cycloheximide per ml) was added, and the plates were incubated for 40 to 48 h at 37°C in 5% CO_2. The cells were fixed with methanol and stained with the *C. trachomatis*-specific fluorescein isothiocyanate-labeled MAb CF-2 (10). Fluorescein-labeled inclusions were counted, and the percent reduction of infectivity was calculated as follows: percent reduction = IFU of control − IFU of test/IFU of control, where IFU is inclusion-forming units, control is an irrelevant antibody and test is the specific MAb or human serum in question. Neutralization was considered positive if a 50% or greater reduction in infectivity occurred. Each assay was performed twice with good agreement in all cases. Neutralization values at the highest MAb or serum concentration were indicated in Tables 1 and 2.

Neutralization with MAbs. The MAbs DP-1 and PE-5 were each tested for their ability to neutralize the serovar or serovariants they recognize in the microtiter plate serotyping method. The MAb DP-1 was shown to recognize only the Da serovariant and not the closely related D, D^-, or D* organisms (Table 1). This MAb neutralized Da serovariants in a dose-dependent manner (data not shown) in the complement-independent assay (Table 1). Furthermore, this MAb neutralized both Da strains tested (MT199 and TW448), but not the D serovar or the D^- or D* serovariants (data not shown). MAb

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TABLE 1. MAbs and their reactivities and neutralizations

MAb	Serovar(s) recognized[a]	Serovariant (strain)	Neutralization result[b]
DP-1	Da	Da (MT199)	+ (87.9)
		Da (TW448)	+ (91.3)
		D	− (0)
		D^-	− (0)
		D*	− (7.7)
PE-5	I, Ia	I	+ (88.7)
		Ia (MT165)	− (49.5)
		I^-	− (0)

[a] Determined by reaction with inclusions (11).
[b] Symbols: +, >50% reduction in infectivity; −, <50% reduction in infectivity. Numbers in parentheses are the average neutralization percentages at the highest MAb concentration.

PE-5 recognized both the I and Ia organisms and not the closely related I^- variant (Table 1). This MAb efficiently neutralized the I serovar in a dose-dependent manner but only weakly neutralized the Ia serovariant and did not neutralize the I^- variant (Table 1).

Neutralization with human immune sera. Each serum sample was diluted and tested against the strain representing the *C. trachomatis* serovar or variant isolated from the patient at the time of culture. The same dilutions of sera were then tested for their ability to neutralize infectivity of closely related serovars or variants to which the patient had presumably not been exposed. Four categories of serological reactivity were identified as shown in Table 2. The first group (represented by serum samples from patients 1 and 2) neutralized both the infecting prototype strain and all of the variants. The second group (represented by sera from patients 3, 8, 9, and 10) failed to neutralize either the infecting organism or any of the closely related strains. The third group (sera from patients 4, 5, and 6) neutralized the infecting strain but did not neutralize one or more of the variants. Finally, one serum sample, a sample from patient 7, failed to neutralize the patient's infecting serovariant but did show neutralizing activity against the standard serovar.

The serovariants examined in these studies were originally identified because they differed from the prototype strains in their MAb typing reactions (13). We determined their MOMP nucleotide sequences and found that they differed at only a few positions from the prototype strains. By analyzing the sequence changes and MAb reactivities, we postulated that the MAbs probably reacted with one VD in which the variants had undergone single amino acid changes from the prototype serovars.

We showed that MAb DP-1 reacts only with the Da serovariant and not with the closely related D serovar or D^- and D* serovariants in the microtiter plate serotyping assay. One Da strain (TW448) differs from the D serovar in the MOMP VD I DNA sequences, but the other Da strain (MT199) had a nucleotide sequence identical to that of the D serovar in VD I (8). Both Da variants, however, had undergone nucleotide changes in VD IV, resulting in a threonine-for-alanine substitution at amino acid position 312 compared with the D serovar sequence. Since both of these Da variants react with DP-1, the changes in VD IV, and not VD I, are probably involved in DP-1 recognition. Furthermore, none of the other D group organisms (D, D^-, and D*) encode a threonine at position 312 in VD IV, and none react with DP-1. DP-1 in fact neutralized both Da strains equally well and did not neutralize D, D^-, or D*, suggesting that this residue is important in DP-1 neutralization. Batteiger (2) recently confirmed the importance of the threonine residue in DP-1 recognition by reacting MAb DP-1 with overlapping synthetic peptides spanning Da serovariant VD IV sequences. He found that DP-1 reacted with the minimal sequence TGTEG in the Da VD IV sequence and did not react with TGAEG found in the VD IV sequence of the other D group organisms (2). Apparently the threonine at position 312 is absolutely required for both DP-1 binding and neutralization because the absence of this residue completely abrogates both recognition and neutralization.

Our results suggest that MAb PE-5 is more flexible in its recognition sequence. This MAb recognizes both the I serovar and Ia variants but not the closely related I^- variants. We found that Ia and I^- variants differed from the I serovar in all four MOMP VDs. However, both these variants had identical sequence changes in VDs II, III, and IV and yet PE-5 reacted with the Ia but not I^- variants. Furthermore, PE-5 recognizes the Ia variant strains MT165 and NL1540 equally well, but NL1540 has the same VD II sequences as the I serovar and MT165 differs from I at position 152 in VD II. Thus, the changes in VDs II, III, and IV may not be important in PE-5 recognition. The sequence changes in VD I, however, indicate that this VD may be involved in PE-5 recognition and neutralization. Both the Ia and I^- variants differ from the I serovar at

TABLE 2. Neutralization assay results using polyclonal immune sera

Patient (infecting serovar or serovariant)	Neutralization result[a] with serovar or serovariant:						
	D	Da	D*	D^-	I	Ia	I^-
1 (D)	+ (76.5)	+ (66.3)	+ (87.5)	+ (96.5)			
2 (D)	+ (70.5)	+ (73.7)	+ (81.0)	+ (85.5)			
3 (D)	− (17.0)	− (33.5)	NT	NT			
4 (D)	+ (77.0)	− (23.2)	+ (57.0)	IR			
5 (D)	+ (61.5)	− (14.5)	− (0)	− (37.0)			
6 (D)	+ (80.5)	− (35.5)	− (49.5)	IR			
7 (D^-)	+ (64.0)	− (33.3)	− (9.0)	− (24.5)			
8 (Ia)					−	− (34.0)[b]	−
9 (Ia)					− (4.9)	− (10.0)	− (32.0)
10 (Ia)					− (4)[b]	− (14)[b]	− (8.0)

[a] +, >50% reduction in infectivity; −, <50% reduction in infectivity; NT, not tested; IR, inconclusive results. Numbers in parentheses are the average neutralization percentages at the highest serum concentration.
[b] Only one assay performed.

position 71 of VD I. Ia variants have an isoleucine-for-valine substitution and I^- variants have a threonine-for-valine substitution at this position. The isoleucine substitution in Ia variants may still allow PE-5 binding, but the threonine substitution in I^- variants may prevent PE-5 binding. Even though the Ia isoleucine substitution at position 71 in VD I did not affect PE-5 binding, it did affect its ability to neutralize Ia variants (Table 1). PE-5 weakly neutralized the Ia variant (49% neutralization at 50 μg/ml) and could not neutralize this organism at higher dilutions, while PE-5 neutralized serovar I organisms strongly at 50 μg/ml but also at the higher dilutions. Thus, PE-5 probably binds to sequences in VD I and is affected in its neutralization but not recognition properties by the substitutions in VD I.

Utilizing sera from infected patients, we observed several patterns of neutralization. Some patients (patients 1 and 2 being examples) produced antibodies that neutralized infection of tissue culture cells both by a representative of their infecting serovar and by all of the closely related D group organisms. Thus, either these patients produced antibodies against portions of MOMP not altered by the sequence changes in the variants or they produced antibodies against multiple MOMP epitopes, at least one of which was not changed in the variants.

Jones and Van Der Pol (7) recently reported a lack of correlation between serum antibodies and the ability of sera to neutralize *C. trachomatis* in vitro. Similarly, some of our patients (patients 3, 8, 9, and 10) had anti-*C. trachomatis* antibodies, but their sera did not neutralize infectivity of *C. trachomatis* for HaK cells. Since these four sera had MIF antibodies ranging from 1:128 to 1:512, we also found that there was not a strict correlation between MIF titers and neutralization.

The third category of neutralization response is the most provocative. These patients' sera (patients 4, 5, and 6) neutralized a representative of their infecting strain but did not neutralize one or more of the closely related D variants. These results suggest that only one epitope in MOMP is recognized by these human immune sera, and when its sequence is altered, the antibodies in these sera are no longer able to neutralize the variant.

The last serum sample we tested (patient 7) was not able to neutralize a representative of the patient's infecting strain (D^-) but was able to neutralize the D serovar. This patient's strain may have undergone a mutation in MOMP which changed its sequence to that of a D^- serovariant, permitting it to escape neutralization by the patient's antibodies. Alternatively, this patient may have originally been infected with a D serovar strain, raised antibodies against this organism which helped eliminate the infection, and then may subsequently have been infected with a D^- serovariant just prior to our *Chlamydia* culture. Again, these results suggested that this patient recognized only one neutralizing epitope, that this epitope is located within MOMP, and that, if its sequence is altered, the antibodies can no longer neutralize the variant.

In summary, we have shown that *C. trachomatis* strains with single amino acid alterations in MOMP VDs escape neutralization with both MAbs and human immune serum. These results suggest that a vaccine strategy based on a MOMP epitope from an individual *C. trachomatis* serovar could fail if a patient is infected with a variant having a single amino acid change within the immunizing MOMP epitope. Further studies are needed to identify conserved MOMP epitopes that exhibit little sequence variation and induce cross-serovar neutralizing antibodies. These results also support the importance of determining how frequently changes in MOMP VDs occur in clinical isolates in a community over time.

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