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Characterization of a Neutralizing Monoclonal Antibody Directed at Variable Domain I of the Major Outer Membrane Protein of *Chlamydia trachomatis* C-Complex Serovars

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A monoclonal antibody (MAb), C10, that neutralized in vitro the infectivity of serovars C, I, J, and L3 (members of the C and C-related complexes) of *Chlamydia trachomatis* was identified. Of the 15 major serovars and the mouse pneumonitis strain of *C. trachomatis*, *Chlamydia psittaci*, and *Chlamydia pneumoniae*, which were used as nontreated and heat-treated (56°C, 30 min) antigens in a dot blot assay, only serovars C, I, J, and L3 were recognized with both the native and treated antigens. Western blot (immunoblot) results showed that MAb C10 recognized the major outer membrane protein of these four serovars. Overlapping hexameric peptides corresponding to variable domains (VDs) I, II, III, and IV of the major outer membrane protein of *C. trachomatis* serovar C were synthesized, and peptide screening showed that MAb C10 mapped to the VD I amino acid sequence VAGLQNDPT. Results of an in vitro neutralization assay correlated with those of the indirect immunofluorescence assay, Western blot, and dot blot assay in that only serovars C, I, J, and L3 were neutralized by MAb C10. In vitro competitive neutralization experiments, using a peptide representing VD I of serovar C to compete with *C. trachomatis* serovar C for MAb C10 binding, revealed that both serological and neutralizing activities of MAb C10 were inhibited by the VD I peptide. In an in vivo toxicity/infectivity assay using serovar L3 pretreated with MAb C10, there was 100% survival of mice infected with a lethal dose at 48 h. In contrast, the control group, consisting of mice injected with the same dose of L3 pretreated with a MAb that does not recognize L3, had no survivors during a 48-h observation period. In summary, since the surface-exposed contiguous epitope recognized by MAb C10 binds neutralizing antibodies that are subspecies specific for the C and C-related complexes, it should be considered for inclusion in the development of a chlamydial vaccine.

Chlamydia trachomatis, an obligate intracellular bacterium, is a major human pathogen responsible for ocular and genital infections. Trachoma, caused by *C. trachomatis* serovars A, B, and C, is the leading cause of preventable blindness in the world, especially in developing countries, while other serovars are more commonly implicated as a cause of genital infections in the Western world (18, 19, 27). Studies of *C. trachomatis* infections in humans and experimental animals provide evidence that partial protective immunity develops following a chlamydial infection (9, 16, 25, 28). Attempts to prevent trachoma by vaccination with the whole organism, however, led only to serovar-specific and short-term protection, and in some cases hypersensitivity and more severe disease upon challenge were reported (9, 25). Therefore, in an attempt to avoid hypersensitivity reactions and to broaden the serovar spectrum of potential vaccines, attention has been focused on the development of a subunit vaccine. As part of this development it is important to characterize potentially protective epitopes. The major outer membrane protein (MOMP), which is surface exposed and contributes 60% of the outer membrane by weight, has been shown to elicit neutralizing antibodies and to contain T-helper cell epitopes (3, 4, 24). If protective epitopes are to be included in a subunit vaccine, the more contiguous and less dependent they are on conformation, the more practical this type of construction will be. Therefore, much attention has been directed at this protein with the goal of identifying contiguous epitopes that may elicit a protective T- and/or B-cell response.

DNA sequencing has revealed that the MOMP contains four variable domains (VDs) I to IV, interspaced with highly conserved regions (21). Information to date has shown that all neutralizing monoclonal antibodies (MAbs) described map to VD regions of the MOMP and that all epitopes to which the neutralizing MAbs are directed are surface exposed on the native organism (12, 14, 30, 31). The majority of neutralizing MAbs described are type specific; however, both MAb E4 described by Peterson et al. (12, 14) and D3 AIII reported by Zhang et al. (31) recognize a conserved region within VD IV and exhibit broad B-complex-neutralizing activity. MAb E4 has also been shown to be contiguous in that a linear peptide representing VD IV is able to compete with infectious *C. trachomatis* for MAb E4 binding (12). To date a contiguous epitope that can bind to a MAb which is able to neutralize multiple members of the C complex has not been described. In this report we present the identification and characterization of a MAb that recognizes a contiguous epitope and neutralizes three of the five C-complex serovars as well as serovar L3 in the C-related complex.

MATERIALS AND METHODS

Organisms. The *C. trachomatis* serovars and strains used in this study were A (G-17), B (HAR-36), Ba (Apache 2), C (TW-3), D (IC-Cal 7), E (Bour), F (UW-6), G (UW-57), H (UW-4), I (UW-12), J (UW-36), K (UW-31), L1 (440), L2

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(434), L3 (404), and mouse pneumonitis (Nigg II). *Chlamydia psittaci* (Texas turkey) and *Chlamydia pneumoniae* (TWAR-183) were also used. All chlamydial isolates were raised in HeLa 299 cells or McCoy cells and frozen at −70°C in 0.2 M sucrose–0.02 M sodium phosphate (pH 7.2)–5 mM glutamic acid (SPG).

MAbs. Elementary bodies of *C. trachomatis* serovar C that were used for the mouse immunizations were raised in HeLa cells and enriched by centrifugation through Renografin as previously described (4). Six- to eight-week old female BALB/c mice (Simonsen Laboratories, Gilroy, Calif.) were injected intraperitoneally with 10^7 inclusion-forming units (IFU) in complete Freund's adjuvant on day 0 followed by the same number of IFU given intraperitoneally in incomplete Freund's adjuvant on day 14. This was followed by an intravenous injection of the same infectious dose in phosphate-buffered saline (PBS; 0.01 M, pH 7.2) on day 21. Three days later the animals were sacrificed and the spleen was removed for a cell fusion that was performed as previously described (14). Hybridoma supernatants were screened by indirect immunofluorescence assay (IFA) (13) and enzyme-linked immunosorbent assay (ELISA). ELISA plates for screening the fusion plates were coated by incubating at 37°C for 1 h with 0.1 ml of a 10-μg/ml suspension of Renografin-purified serovar C elementary bodies in PBS per well. Plates were then blocked with super cocktail, a 1% solution of both bovine and chicken albumin in PBS–0.05% Tween, for 1 h at 37°C. Plates were washed with PBS–0.05% Tween and stored dried at −20°C until used. Culture fluid from individual wells, 0.05 ml, was then added to the ELISA plates and incubated for 1 h at 37°C, and then plates were washed five times with PBS-Tween. The second antibody, goat anti-mouse immunoglobulins labeled with horseradish peroxidase (Cappel, Durham, N.C.), was added, and the ELISA plates were incubated for 1 h at 37°C and then washed by the same protocol as before. Subsequently, 0.1 ml of a 1 mM ABTS solution (Sigma, St. Louis, Mo.), 2,2′-azino-bis(3 ethyl-benzthiazodinsulfonate) dissolved in ABTS buffer (0.1 M disodium P_i, 0.08 M citric acid, pH 4.0), was added, and color was allowed to develop for 20 min, after which time wells were inspected visually and in some cases read by a microtiter reader at 405 nm (Bio-Rad Laboratories, Richmond, Calif.).

Hybridomas positive by either of these two assays were further tested by an in vitro neutralization assay. Neutralizing hybridomas were selected and cloned by limiting dilution. Ascitic fluid was produced by injecting 10^6 hybridoma cells intraperitoneally into 4- to 6-week-old BALB/c mice that had been injected intraperitoneally with pristane 10 days previously. Immunoglobulin was purified from mouse ascitic fluid by using the Affi-Gel protein A MAPS II MAb purification system (Bio-Rad). Purified MAbs were stored in PBS at −70°C.

Immunoassays. Western blots (immunoblots) were performed as described previously (5), with the exception that a tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis system was used (20). The dot blot assay utilizing untreated and heat-treated (56°C, 30 min) elementary bodies was performed as previously described by Zhang et al. (30). An inclusion IFA was done as described previously (13).

Peptide mapping. Overlapping hexapeptides were synthesized according to the method of Geysen et al. (7, 8) using a commercially available epitope-mapping kit (Cambridge Research Biochemicals, Cambridge, England). Peptides synthesized corresponded to the four VDs of the MOMP of serovar C and the VD I of serovars A, H, I, J, K, and L3. The immobilized peptides were assayed by ELISA as previously described (12).

In vitro neutralization assays. The in vitro neutralization assays used in this report have been previously described (12, 14). Briefly, MAbs were diluted in PBS containing 5% guinea pig serum (GPS; Whittaker M. A. Bioproducts, Walkersville, Md.). *C. trachomatis* 4×10^4 IFU, was added to the MAb dilutions and to controls which consisted of 5% GPS in PBS without MAb. The mixtures were incubated at 37°C for 45 min and inoculated in duplicate onto confluent HeLa cell monolayers contained in glass vials (15 by 45 nm), which were washed twice with PBS immediately before inoculation. Cells were infected by centrifugation, followed by stationary incubation at 37°C for 1 h, after which time cultures were fed with 1 ml of Eagle's minimal essential medium containing Earle's salts, 10% fetal bovine serum, gentamicin (50 μg/ml), and cycloheximide (1 μg/ml). Infected monolayers were incubated, fixed, and stained with MAb E4 directed to VD IV of the MOMP as previously described (12). A total of 10 × 200-magnified fields per coverslip culture were counted. On the average, the controls gave 23 IFU (standard deviation, ± 8) per × 200-magnified field. MAbs yielding values that were <50% of the control IFU values (>50% reduction in IFU of control) were considered positive for neutralization. Each test was repeated a minimum of three times.

A 21-amino-acid peptide representing VD I of serovar C with an additional C at the N terminus, CAAPTTSDVAGL QNDPTTNVA, was used in a competitive neutralization assay with serovar C. A 16-amino-acid peptide, ATAIFDTT TLNPTIAG, representing 16 amino acids in VD IV of serovar E was used as a control peptide. All control assays were the same as the test reactions except that MAb E23, which does not recognize serovar C, was substituted for MAb C10. This assay was performed as previously described (12). A paired *t* test was used in analyzing the data from the competitive neutralizations.

In vivo toxicity/infectivity. An in vivo toxicity/infectivity assay was performed as described previously (14). Briefly, 5×10^8 IFU of *C. trachomatis* serovar L3 were mixed with 100 μg of MAb C10 in 5% GPS. The control mixture consisted of the same infectious dose of serovar L3 with 5% GPS and MAb E23, which does not neutralize *C. trachomatis* infectivity in vitro. The mixture was incubated at 37°C for 45 min. After incubation, the mixtures were injected into the tail vein of 4- to 6-week-old female BALB/c mice. Both the MAb C10 group and the MAb E23 group consisted of six mice. Animals were observed daily for up to 3 weeks and scored for death.

RESULTS

MAb C10, of the immunoglobulin G2a isotype, recognized serovars C, I, J, and L3 when tested by an inclusion IFA using the mouse pneumonitis strain and the 15 serovars of *C. trachomatis*, *C. psittaci*, and *C. pneumoniae*. Also, as is shown in Fig. 1, in a dot blot assay using both nontreated and heat-treated (56°C, 30 min) antigens, only serovars C, I, J, and L3 were recognized. Corroborating the inclusion IFA and dot blot results, only the MOMPs of serovars C, I, J, and L3 were positive by Western blot (Fig. 2).

To locate the epitope on the MOMP recognized by MAb C10, overlapping hexameric peptides representing the four VD regions of serovar C were synthesized and used in an ELISA. MAb C10 reacted only within VD I to the peptides spanning the sequence VAGLQNDPT, with the critical

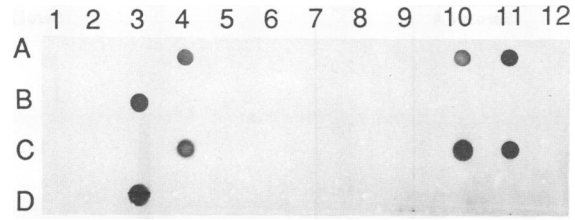

FIG. 1. Dot blot of nontreated (rows A and B) and heat-treated (rows C and D) chlamydiae cells reacted with MAb C10 (10 μg/ml). Columns in rows A and C contain *C. trachomatis* serovars as follows: 1, A; 2, B; 3, Ba; 4, C; 5, D; 6, E; 7, F; 8, G; 9, H; 10, I; 11, J; and 12, K. Columns in rows B and D contain the following: 1, L1; 2, L2; 3, L3; 4, HeLa cell control; 5, mouse pneumonitis strain; 6, *C. pneumoniae* (TWAR-183); 7, *C. psittaci* (Texas turkey).

residues being GLQND (Fig. 3). This is also the same sequence in serovar J. The VD I regions of the other serovars in the C and C-related complexes were also synthesized and tested with MAb C10. As is illustrated in Fig. 3, MAb C10 failed to recognize the overlapping peptides to the corresponding A serovar sequence which had two amino acid substitutions (underlined), GLEKD, within the critical binding region. The hexapeptides representing serovar H with three substitutions, AADLQNDPK, one inside the critical binding area, were recognized to a lesser extent than the other serovars that were positive with MAb C10 in a dot blot. When MAb C10 was tested with the sequences representing serovars L3 and I which each had one substitution, GLSND (L3) and GLEND (I), within the MAb C10 critical binding region, the reactivity appeared similar to that of serovar C. The VD I region of serovar K that corresponds to the MAb C10 recognition site in serovar C had a single substitution outside the prime binding residues and had reactivity in the peptide scan similar to that of serovar C. This finding is interesting in that the K serovar, when tested with MAb C10, was negative by all other serological assays performed.

When MAb C10 was tested in an in vitro neutralization assay against serovars in the C (serovars A, C, H, I, and J), C-related (serovars L3 and K), B (serovars E and L2) and B-related (serovar F) complexes, only those serovars that were recognized in the dot blot, Western blot, and IFA were neutralized by MAb C10 (Fig. 4). Although the peptides

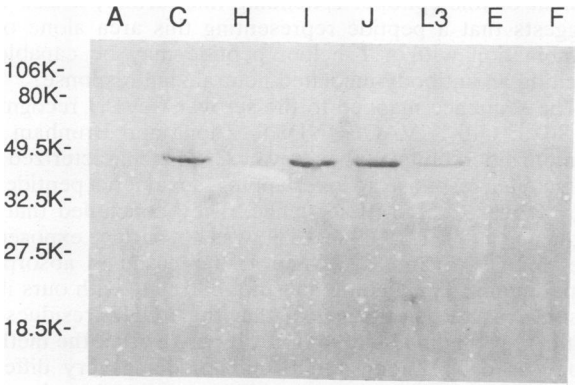

FIG. 2. Western blot using MAb C10 (10 μg/ml) to probe eight *C. trachomatis* serovars, representing the C (serovars A, C, H, I, and J), C-related (serovar L3), B (serovar E), and B-related (serovar F) complexes.

representing the VD I peptides of serovar H exhibited weak activity with MAb C10, no neutralization of serovar H was detected at the concentrations of antibody tested. The amounts of MAb C10 to achieve the 50% neutralizing endpoints for serovars C, I, J, and L3 were 0.50 ± 0.18, 18 ± 9.40, 0.95 ± 0.48, and 12 ± 6.01 μg/ml, respectively.

To demonstrate whether a 21-amino-acid peptide representing VD I of serovar C was able to compete with *C. trachomatis* organisms for MAb C10 binding, a competitive neutralization assay was performed (Fig. 5). A 16-amino-acid peptide made to serovar E VD IV was used as a negative control. In the assay different concentrations of MAb C10 were mixed with 40 μg of each peptide per ml. While the percent reduction in IFU was the same for MAb C10 with or without the control VD IV peptide, the results in the presence of the VD I peptide showed competition at 0.5 and 1 μg of MAb C10 per ml ($P < 0.01$). The amount of MAb C10 (1.2 μg/ml) needed to achieve 50% reduction in serovar C IFU in the presence of the VD I peptide was over twice the 0.5 μg/ml required without the peptide.

To test MAb C10 for its ability to neutralize chlamydial toxicity/infectivity in vivo, *C. trachomatis* serovar L3 was pretreated with MAb C10 and 5% GPS at 37°C for 45 min before being injected intravenously into mice. MAb E23 directed at the MOMP of serovar E, which does not recognize serovar L3, was used as a control. All six mice that received L3 pretreated with MAb E23 died within the first 48 h. In contrast, the six animals that received L3 preincubated with MAb C10, except one which died at 72 h, survived beyond 3 weeks.

DISCUSSION

There is evidence that whole organisms of *C. trachomatis* are an unsuitable vaccine candidate because of their ability to induce a hypersensitivity reaction and their serovar restriction in terms of protection (9, 25, 28). For these reasons, investigators have been focusing on identifying epitopes of *C. trachomatis* that are able to elicit host protection with the goal of developing a subunit vaccine. Work with animal models of chlamydial infections has shown that both arms of the immune system may contribute to a host protective response (2, 17, 29). Therefore, in order to develop a subunit vaccine that is able to elicit a broad level of protection for all serovars, identification of several epitopes, both those that stimulate the cellular arm and those that stimulate the humoral arm of the immune system, is important. To this end the VD IV region of the MOMP has been shown to elicit a B-complex-neutralizing antibody response (1, 6, 22, 26). Two B-complex-neutralizing MAbs, resulting from mice immunized with whole organisms of *C. trachomatis*, have been described. These MAbs, E4 and DIII A3, recognize a similar peptide within the conserved nonapeptide TTLNPTIAG in VD IV of the MOMP (11, 12). A 16-amino-acid peptide representing VD IV of the MOMP is able to compete with infectious *C. trachomatis* for MAb E4 binding as determined by an in vitro neutralization assay, suggesting the functional epitope to be contiguous (12). Although the region recognized within the VD IV is conserved among the B- and C-complex serovars, in work to date with BALB/c mice, it fails to elicit a strong neutralizing response to the C-complex serovars (6, 26). Therefore, information on contiguous epitopes that neutralize a broad number of C and C-complex serovars is lacking.

This is the first report of a MAb that maps to a contiguous region within VD I of the MOMP that is able to show broad

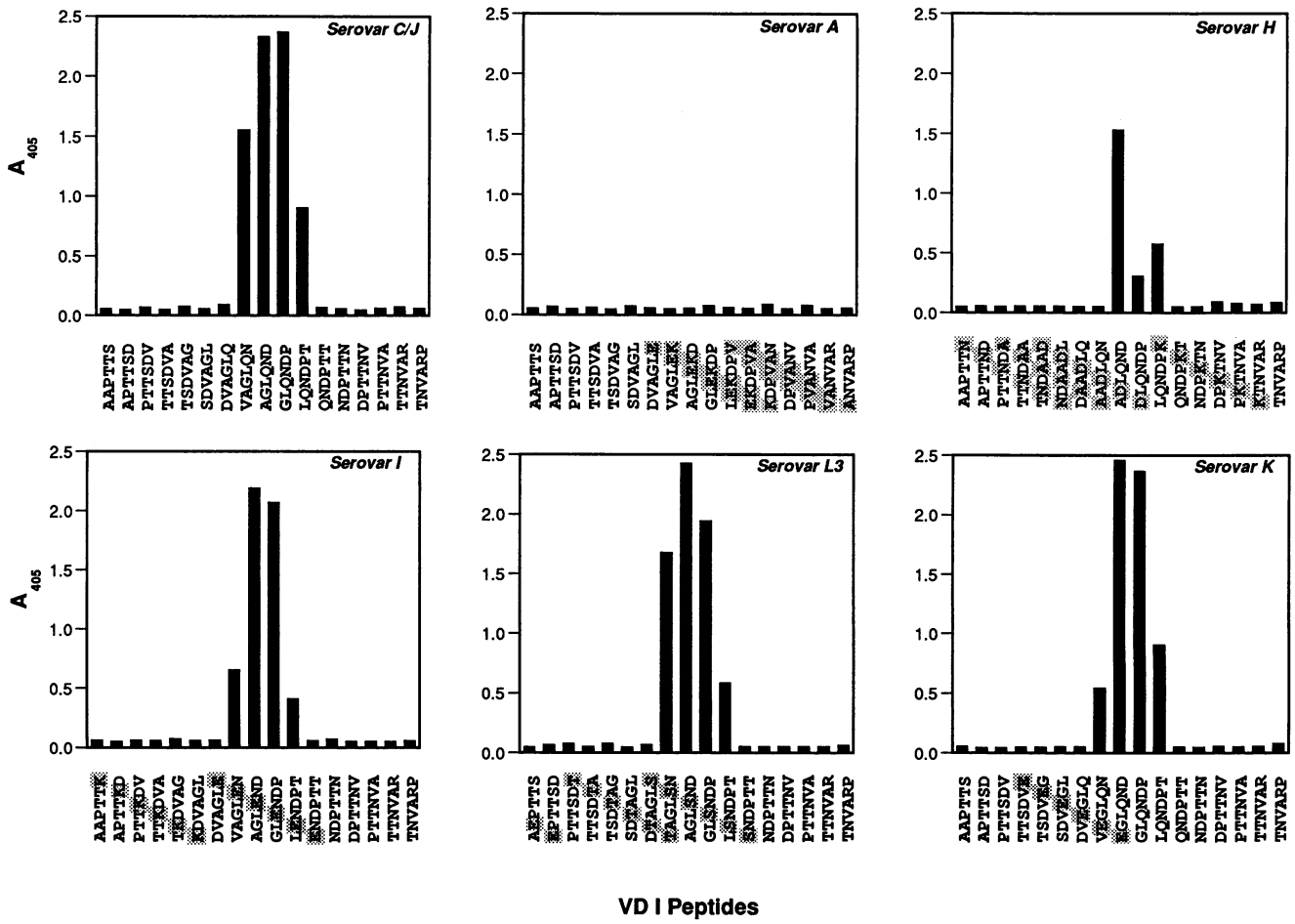

FIG. 3. Results of ELISA of MAb C10 with synthetic overlapping hexameric peptides corresponding to the MOMP VD I regions of *C. trachomatis* serovars A, C, H, I, J, K, and L3. MAb C10 was tested at a concentration of 10 μg/ml with a 20-min substrate incubation. Shaded amino acids represent those that differ from the serovar C amino acid sequence.

neutralizing activity within the C complex. Other MAbs that neutralize within the C complex appear to be serovar specific in both their serological activity and neutralizing ability (10, 30, 31). An exception to this is MAb HV-A5, described by Zhang et al. (31), which protects mice in an in vivo toxicity assay from serovars C and H; however, this determinant has not been defined by epitope-mapping studies. It is interesting that MAb C-28, which has been described by Morrison and colleagues (11), recognizes the sequence DVAGLQND within VD I and is C serovar restricted in recognition and neutralization. MAb A-20, reported by the same investigators, is restricted in recognition and neutralization to the A serovar and binds to the VD I epitope DVAGLEKD (11). This in contrast to MAb C10, reported here, that recognizes a similar sequence, VAGLQNDPT, and yet is more broadly reacting in terms of recognition and neutralization, in which serovars C, I, J, and L3 are bound and neutralized. Equally important is the fact that a linear peptide representing the VD I region of serovar C is able to compete with infectious *C. trachomatis* for MAb C10 binding. While there was competition between the peptide and infectious *C. trachomatis* for MAb C10 binding, it is clear from the results that it was not as efficient as *C. trachomatis* in binding MAb C10. This was also our experience in competition assays in which a peptide representing VD IV of serovar E was used to compete with infectious *C. trachomatis* for MAb E4 binding (12). Therefore, the actual epitope in the intact organism may be slightly different from that seen in a peptide scan assay using hexameric peptides. However, because there was competition with the peptide, this suggests that MAb C10 recognizes a functional, i.e., neutralizable, linear component of this epitope. Extending this further, it also then suggests that a peptide representing this area alone or in combination with a T helper peptide may be capable of eliciting an antibody-mediated neutralizing response.

The sequence mapped to the serovar C VD I recognized by MAb C10 is VAGLQNDPT. Zhong and Brunham (32) immunized rabbits with serovar C and characterized the polyclonal response to overlapping hexameric peptides of the serovar C MOMP sequence. They concluded that the sequence in VD I, LQNDPTTN, was not surface exposed on the three serovars, C, I, and J, they used in absorption experiments. This finding does not correlate with ours if we assume, as the data suggest, that the LQND residues are critical for binding by MAb C10. It may be that the methodology used by Zhong and Brunham, being very different from the approach we presented here, contributed to this difference, and the different responses in rabbits versus mice in terms of the specificity of antibodies produced may also contribute to the conflicting results. So far our data suggest-

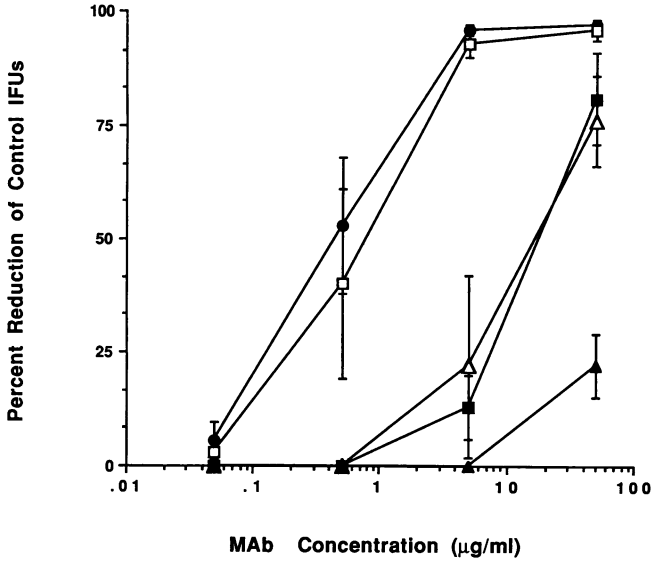

FIG. 4. In vitro neutralization assay of MAb C10 with serovars C (●), J (□), I (■), L3 (△), and H (▲) of *C. trachomatis*. Control assays were performed in the same way as the MAb C10 reactions except that MAb C10 was omitted. Each point is the average of values from at least three separate experiments. The bars represent the standard deviations.

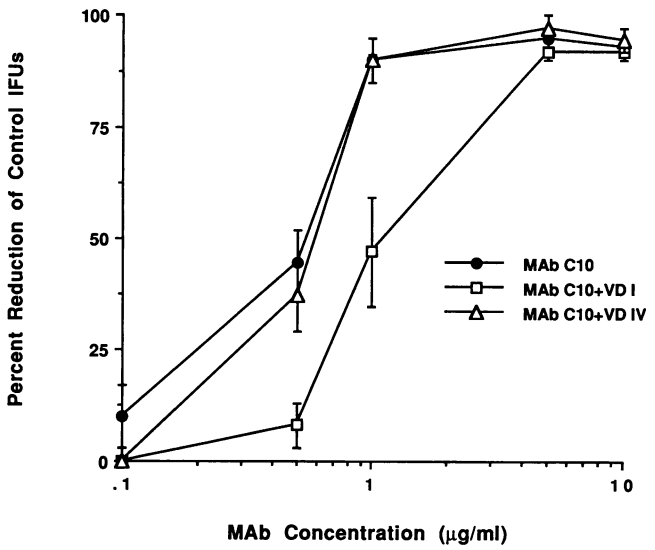

FIG. 5. Competitive in vitro neutralization of *C. trachomatis* serovar C with a peptide, CAAPTTSDVAGLQNDPTTNVA, representing VD I of the MOMP. The control peptide, ATAIFDTTTL NPTIAG, represents 16 amino acids within VD IV of serovar E. MAb E23, which did not neutralize *C. trachomatis* serovars, was used as the control and as with MAb C10 was assayed in the presence of the VD I and the VD IV peptides. The bars represent the standard deviations.

ing that the epitope recognized by C10 is surface exposed are consistent with our previous findings and those of others that only surfaced-exposed antigenic determinants of the MOMP elicit protective antibodies (1, 12, 14, 30–32).

Analysis of the patterns of reactivity of the overlapping hexameric peptides representing the VD I regions of the C- and C-related-complex serovars showed that substitutions of critical residues within the serovar C recognition sequence abrogated both serological and functional (i.e., neutralizing) activity of MAb C10. In our experiments, substitution of Q with E or S, while it attenuated binding of serovars I and L3, respectively, to some degree, did not abolish recognition or neutralization. The underlined substitutions in serovar A, VAGLEKDPV, completely abolished recognition by MAb C10. This finding is corroborated by the work with MAb A-20 which shows that these same two amino acid substitutions, EK, in this epitope restrict MAb binding and neutralization to serovar A within the C complex (11). Serovar H, AADLQNDPK, with a substitution in the region GLQND which appears to be necessary for optimal binding by MAb C10, gave weak binding in the ELISA with the hexameric peptides and was negative in all other serologic and neutralization assays. Interestingly, serovar K with one substitution at the N terminus of the peptide, VEGLQNDPT, recognized the hexameric peptides representing the MAb C10 binding region to a similar extent as the serovar C sequence. However, serovar K, like serovar H, showed no recognition of MAb C10 in assays of whole organisms or denatured serovar K. In the dot blot of untreated serovar K it appears that the lack of recognition by MAb C10 was due to the fact that the epitope was not surface accessible. However, this may not be the full explanation since in the Western blot, where theoretically exposure should not be a problem, there still was no recognition. Therefore, it appears that other factors may influence the structure of this VD region in serovar K, possibly other amino acid residues in the MOMP that may be in close proximity to the MAb C10 binding region. This illustrates the need for interpreting results by using synthetic overlapping peptides along with other binding and functional assays.

A logical step in the investigation of contiguous neutralizing epitopes is to immunize animals with a peptide containing neutralizing epitope(s) to see whether a similar neutralizing response can be induced. A peptide representing the serovar E VD IV has been reported to elicit a strong neutralizing response against serovar E (6, 26). We have initiated these types of experiments with a peptide representing the serovar C VD I sequence; using this approach we have detected neutralizing antibodies to serovar C, and polyclonal antibody to this peptide maps to the same region within VD I as MAb C10 (15). Su and Caldwell (23) have reported on a chimeric peptide that contained both a T-cell helper peptide and a region that contained the serovar A neutralizing epitope, DVAGLEKD. They too found that this peptide was able to elicit a neutralizing response against serovar A when immune serum was tested in an in vitro neutralization assay. In conclusion, this type of experimental approach has yielded promising results, and the information gained is critical in the overall strategy for the construction of chlamydial subunit vaccines; however, further efforts must be made to identify more protective epitopes if we are to be successful in achieving this goal.

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