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Antibody Recognition of a Neutralization Epitope on the Major Outer Membrane Protein of *Chlamydia trachomatis*

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Two BALB/c mice were immunized with serovar C *Chlamydia trachomatis* elementary bodies, and 63 hybridomas producing monoclonal antibodies to *C. trachomatis* were recovered. Eight hybridomas which were specific for an identical peptide epitope (AGLQND) in serovar C major outer membrane protein variable domain 1 were identified. Detailed immunochemical study of the antigen-antibody interaction and genetic characterization of the antibody variable-region gene sequences showed that distinct B-cell clonal lineages were elicited by the epitope sequence. Since each antibody had a distinct pattern of fine specificity for recognition of the epitope and displayed different degrees of cross-reactivity with a related serovar (serovar A), we conclude that B-cell recognition of an immunodominant neutralization epitope can be pleiotropic. Differences in B-cell recognition of a neutralization epitope may delay the emergence by mutation of antigenic-drift variants of the *C. trachomatis* major outer membrane protein.

The major outer membrane protein (MOMP) of *Chlamydia trachomatis* is specified by a single-copy gene, omp1, which exhibits extensive allelic polymorphism (16). omp1 DNA polymorphism is mainly clustered in regions that specify four variable domains (VDs) which are speculated to be arrayed as immunoaccessible loops on the bacterial cell surface (2). Amino acid sequence variation in the VDs determines the serologic properties which allow classification of *C. trachomatis* into 15 or more serovars (18, 19).

Among experimentally immunized animals, *C. trachomatis* MOMP antibody responses are directed principally to the VDs (21). Furthermore, monoclonal antibodies (MAbs) to VD 1, 2, and 4 are neutralizing in tissue culture and protective in primate models of experimental ocular infection (11). For this reason, synthetic peptides derived from VD amino acid sequences are being developed as candidate oligopeptide vaccines for prevention of *C. trachomatis* infection (17, 24).

The immunochemistry involved in antibody recognition of a VD has not been studied. How antibody recognition of a MOMP VD epitope might select for alternate or variant serovars in a population is also unclear. This may be especially relevant when oligopeptides are used as vaccines, since they can be expected to engender highly focused antibody responses. To evaluate the immunochemical and genetic basis for antibody recognition of a neutralization epitope on the *C. trachomatis* MOMP that may be incorporated into an oligopeptide vaccine, the antibody-antigen interaction of eight MAbs and the antibody variable-region gene sequences were determined. All eight MAbs recognized an overlapping epitope sequence. The data may be relevant to anticipating the immunoepidemiologic effects of an Omp1-based oligopeptide vaccine.

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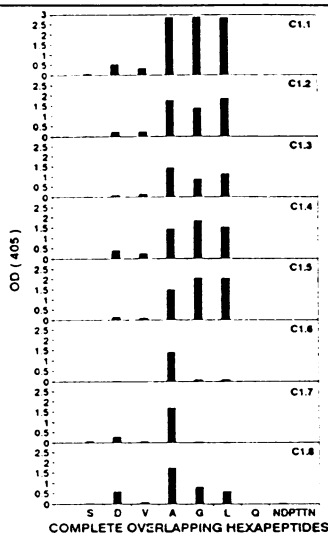
MATERIALS AND METHODS

Murine MAb production. Two female BALB/c mice (4 weeks old) from Jackson Laboratories were immunized with approximately 5×10^5 inclusion-forming units (IFU) of serovar C elementary bodies (EBs), raised and purified as described previously (23) in 50 μl of SPG (sucrose-phosphate-glutamic acid buffer [pH 7.4]) mixed with an equal amount of complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) by intraperitoneal injection. Twenty-one days later, a second injection was given intravenously with the same number of organisms in 50 μl of SPG without adjuvant. An identical intravenous boost injection was given to the mice 7 days after the second injection. Three days after the final injection, spleens were removed aseptically, and a single-cell suspension was made. Splenocytes were fused with NS1 myeloma cells (P3/NS1/1-AG4-1; American Type Culture Collection) in the presence of polyethylene glycol (Sigma Chemical). One week after the fusion, tissue culture fluid was screened for MAbs to *C. trachomatis* serovar C by a dot enzyme-linked immunosorbent assay (ELISA) and antibody specificity was confirmed by staining acetone-fixed 72-h-grown serovar C inclusions in an indirect immunofluorescence assay. Hybridomas secreting *C. trachomatis*-reactive antibodies were expanded and subcloned by limiting dilution. Ascites was produced in 8- to 10-week-old BALB/c mice injected intraperitoneally with 0.5 ml of incomplete Freund's adjuvant 1 week prior to hybridoma cell injection. The MAbs were isotyped with an Immunopure MAb isotyping kit (Pierce) and purified with the Immunopure (A/G) immunoglobulin G (IgG) purification kit (Pierce).

Multiple-pin peptide synthesis and pepscan-ELISA. Peptides were synthesized on solid polyethylene rods with a commercially available kit (Cambridge Research Biochemicals, Cambridge, United Kingdom) (17) by using f-moc chemistry as described before (23). Two sets of peptides were used in this study. The first set includes 370 complete overlapping hexapeptides covering the entire MOMP sequence of serovar C. This set of peptides was used to map the MAb epitopes in a pepscan-ELISA. For mapping the fine specificity of the MAbs, a second set of complete substitution analogs of the sequence VAGLQND in which each residue was sequentially replaced with each of the 19 alternative amino acids were synthesized. Each MAb was reacted with each peptide analog,

TABLE 1. Mapping MAb epitopes

mAb designation		Isotype H(L) Chain	Hexapeptide Binding Pattern[a]	Minimal Length of Peptide[b]
C1.1		IgG3(k)		LQND
C1.2		IgG3(k)		LQND
C1.3	Group I	IgG1(k)		LQND
C1.4		IgG2b(k)		LQND
C1.5		IgG1(k)		LQND
C1.6		IgG1(k)		AGLQND
C1.7	Group II	IgG3(k)		AGLQND
C1.8		IgG3(k)		AGLQND

[a] Each MAb was reacted with a panel of 370 overlapping peptides (see text). Only reactive peptides are displayed along the horizontal axis by the letter of the first amino acid in the peptide.

[b] Determined by MAb reactivity with complete overlapping peptides.

and the optical density at 405 nm (OD_{405}) was used to express the result. For fine-specificity mapping, the result was expressed as a percentage of the OD value for the parent control peptide. If substitution of a peptide epitope residue with more than four amino acids reduced antibody binding to the substitution analog by more than 80% compared with that to the parent peptide epitope, that residue was defined as being critical in the peptide epitope for recognition by a given antibody. The >4-amino-acid substitution cutoff criterion was used to exclude conservative substitutions, and the >80% binding activity cutoff was determined on the basis of the frequency distribution of the antibody-binding OD values for all eight MAbs (data not shown). Amino acid substitution analysis provides a detailed "fingerprint" for the epitope-paratope interaction (24). The rationale for this form of analysis is that if changing a residue in the peptide epitope to almost any other amino acid significantly decreases antibody binding to the peptide analog, then that residue is presumed to be directly involved in the epitope-paratope interaction. Recent elucidation by X-ray crystallography of Fab-peptide interaction has confirmed that nonsubstitutable amino acids in the pepscan format are contacting residues in the crystal structure (20, 22).

Immunoblot and radioimmunoprecipitation. The immunoblot and radioimmunoprecipitation assays were done as described previously (22).

Immunodot blot. The dot blot assay was modified from the procedure originally described by Zhang et al. (20) as follows. Serovar C EBs (2×10^5 IFU) were dotted onto a nitrocellulose membrane in 50 μl of SPG. For heat treatment, EBs were heated at 56°C for 30 min. The membrane was blocked with 4% bovine serum albumin and 0.05% Tween 20 in phosphate-buffered saline (PBS). A mixture of goat anti-mouse IgG conjugated with ^{125}I (ICN Biochemicals) and ^{125}I-protein G (ICN Biochemicals) was used to visualize MAb binding.

ELISA. Microtiter plates (Immulon 2; 96 round-U wells; Dynatech Laboratories, Alexandria, Va.) were coated overnight at 4°C with 10^6 IFU of serovar C EBs in 100 μl of SPG per well. The plates were washed once with 200 μl of SPG and blocked with 200 μl of blocking solution per well at 37°C for 90 min. Serial dilutions of antibody preparations were added to the plates at 100 μl per well. The plates were incubated at 37°C for 60 min. After the plates were washed four times with 200 μl of wash solution (0.05% Tween 20 in PBS [pH 7.4]) per well, a goat anti-mouse IgG-peroxidase conjugate (Pierce) at a 1:4,000 dilution in incubation solution was added at 100 μl per well, and the plates were incubated as before. After the plates were washed, 100 μl of substrate [2,2-azino-bis-(3-ethyl-benzthiazoline-6 sulfonate) in citrate buffer (pH 4.5) in the presence of H_2O_2] was added to each well. The enzymatic reaction was allowed to proceed for 15 min at room temperature, and the absorbance was measured at 405 nm on a Titertek Multiskan plus version 1.43 automatic ELISA reader (EFLAB).

Competition ELISA. The competition ELISA was carried out with serovar C EB-coated plates. Serial dilutions (50 μl) of corresponding competitors (serovar A or C EBs) in SPG were added to the serovar C EB-coated plates, after which 50 μl of a selected dilution of each MAb preparation that yielded an absorbance of approximately half the maximum value in the titration ELISA described above was added. Competition was allowed to proceed for 60 min at 37°C and was terminated by washing away the soluble components. The rest of the steps were performed as described for the standard ELISA. The percent inhibition at each competitor concentration was calculated as [(absorbance without competitor − absorbance with competitor)/absorbance without competitor] × 100. The IFU of EBs required for 50% inhibition ($I_{0.5}$) was determined. The $I_{0.5}$ value measured by the competition ELISA correlates inversely with the affinity value (K; per molar) as measured by

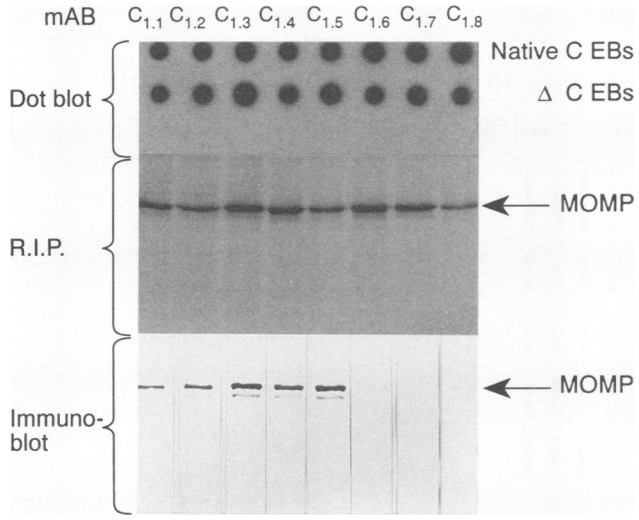


FIG. 1. MAb binding to whole EBs (native or heat treated [△]); in dot blot and to MOMP in radioimmunoprecipitation (R.I.P.) and immunoblot assays. Each antibody was used at a 1:1,000 dilution for all assays.

conventional equilibrium dialysis (22). Therefore, the higher the $I_{0.5}$ value, the lower the antibody affinity.

In vitro neutralization assay. An HAK cell line (hamster kidney cells) in vitro neutralization assay was performed as described before (21). Neutralization assays were done by preparing serial twofold dilutions of purified IgGs in SPG. An MAb with unrelated specificity was similarly diluted and used as a control. Chlamydial inclusions were detected by staining with rabbit anti-chlamydial serovar A, B, and C polyclonal antiserum (1:1,000) as the first antibody, horseradish peroxidase-conjugated goat anti-rabbit Ig antibodies as the secondary antibody, and 1-chloronaphthanol (Sigma Chemicals) as the substrate. IFU were quantitated by counting five fields at a magnification of ×200 with an inverted microscope. A mean IFU per field was calculated from three plates, and the results were expressed as percent reduction in mean IFU compared with the control wells.

RNA and cDNA preparation for variable-region gene sequencing. Total cellular RNA was extracted from hybridoma cells from each clone by acid guanidium thiocyanate-phenol-chloroform extraction (11). Approximately 10^8 cells were grown in RPMI 1640 medium (GIBCO) with 20% fetal calf serum (Intergen), harvested by centrifugation, and washed once with PBS (pH 7.4). The cell pellet was lysed in 5 ml of denaturation solution (4 M guanidinium thiocyanate, 25 mM sodium citrate [pH 7], 0.5% sarcosyl, 0.1 M 2-mercaptoethanol). The protein and DNA were removed by adding 0.1 volume of 0.2 M sodium acetate (pH 4), 1 volume of water-saturated phenol, and 0.2 volume of chloroform. RNA in the aqueous phase was precipitated by adding 1 volume of isopropanol, and the pellet was dissolved in the denaturation solution and reprecipitated as above. The final pellet was washed with 75% ethanol and resuspended in 75% ethanol for storage at −70°C or with 0.1% of diethyl pyrocarbonate (Aldrich)-treated water for further mRNA purification. The mRNA was purified by chromatography on oligo(dT)-cellulose with a Poly(A) Quick mRNA Purification kit (Stratagene, La Jolla, Calif.). First-strand cDNA was prepared with oligo(dT) or specific oligonucleotide primers (see below for the constant-region-specific primers used for PCR amplification) that hybridize to Ig constant-region genes with a Superscript (GIBCO BRL) or a cDNA Synthesis (Pharmacia LKB Biotechnology) kit.

PCR amplification and sequencing. The cDNA samples were amplified by PCR. The amplifications were carried out in

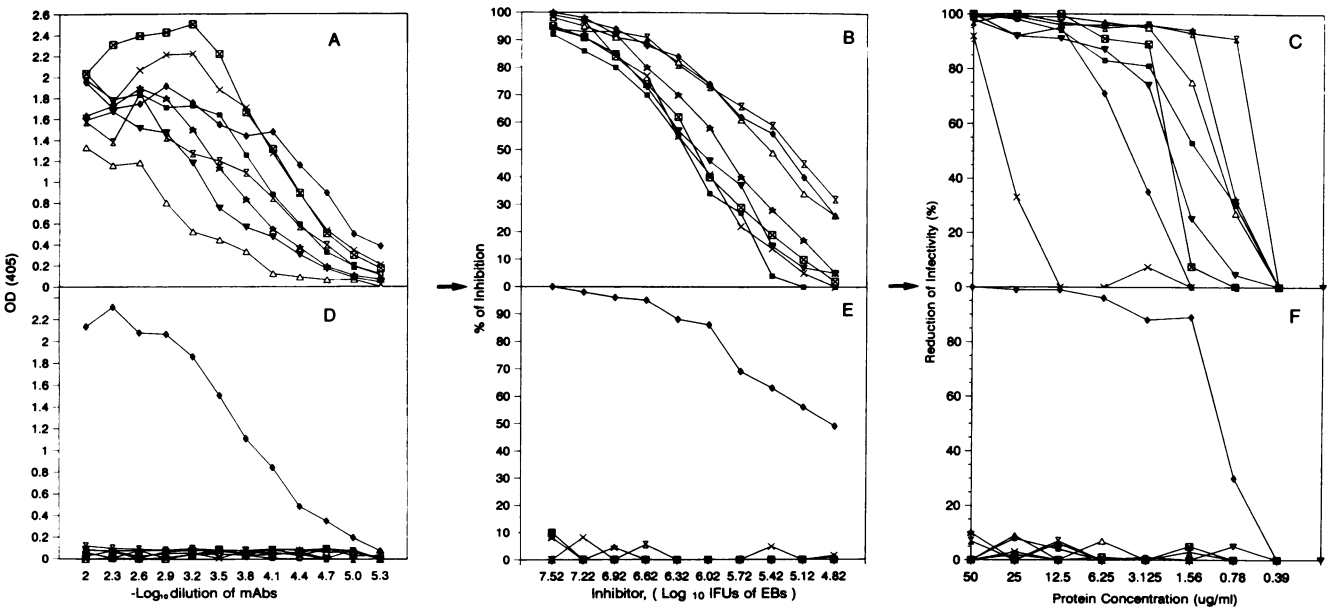


FIG. 2. Comparison of OD values for binding of the eight MAbs to serovar C (A, B, and C) and to serovar A (D, E, and F) in an ELISA (A and D) and a competition ELISA (B and E) and of neutralization of serovars C and A in an in vitro neutralization assay (C and F). In the standard ELISA, the plates were coated with either serovar C (A) or serovar A (D); in the competition ELISA, both plates were coated with serovar C, and either serovar C (B) or serovar A (E) was used as the competitor. ★, C1.1; ▼, C1.2; ☒, C1.3; ■, C1.4; ×, C1.5; ◆, C1.6; △, C1.7; ⧖, for C1.8.

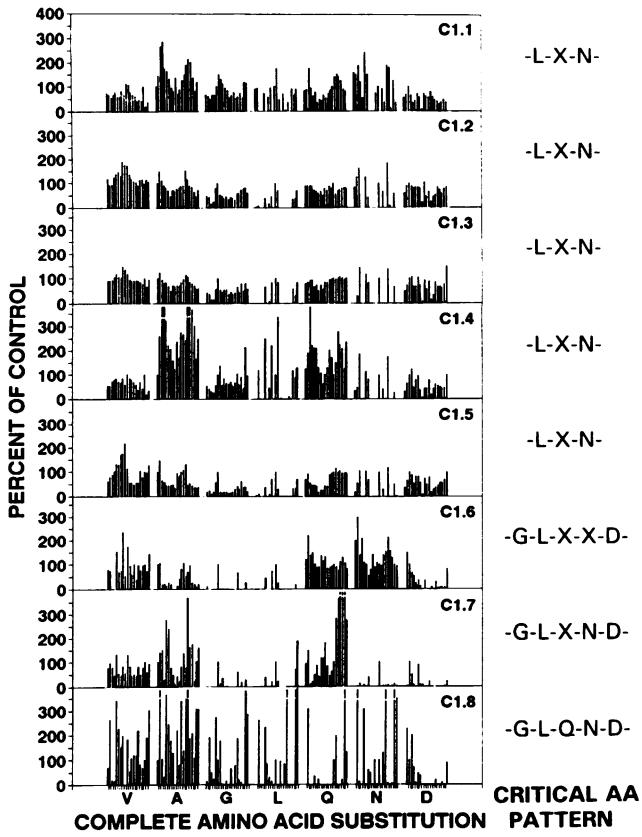


FIG. 3. Fine-specificity mapping with complete substitution analogs of the epitope sequence VAGLQND. MAbs C1.1 to C1.8 were reacted with individual substitution analogs. Analogs for each amino acid position are in alphabetical order in the single-letter code for amino acids. Values represent the binding of the analog as a percentage of that of the parent peptide. Values above 400% of the parent peptide value are indicated with a break in the bar. The critical binding motifs, determined as described in the text, are shown to the right. X stands for any amino acid.

a 100-μl reaction mix containing 10 mM Tris (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 250 μM dATP, 250 μM dCTP, 250 μM dGTP, 250 μM dTTP, 100 ng each of 3′ and 5′ primers, and 2.5 U of *Taq* polymerase (Perkin Elmer Cetus). The reaction samples were placed in boiling water for 5 min before addition of the enzyme. The reaction was allowed to proceed for 35 cycles of 2 min at 72°C, 45 s at 94°C, and 1.5 min at 50°C. The PCR products were sequenced with a double-stranded DNA cycle sequencing system (Bethesda Research Laboratories) with the use of the appropriate ^{32}P-labelled constant-region-specific primer to direct synthesis.

The amplified product of each reaction was checked on a 1% agarose gel and precipitated by adding 0.5 volume of 8 M ammonium acetate and 1.5 volumes of isopropanol and incubating for 10 min at room temperature. The mixture was centrifuged in a microcentrifuge for 10 min at room temperature, and the pellet was washed twice with cold 70% ethanol and evaporated to dryness in a Speed Vac Concentrator. The final pellet was dissolved in an appropriate amount of TE (Tris-EDTA) buffer in preparation for direct double-stranded cycle sequencing. The primers used for sequencing were end labelled in a 20-μl reaction mix containing 200 ng of primer, 4 μl of 5× kinase buffer, 2 μl of (γ-^{32}P)ATP (>7,000 Ci/mmol, 10 mCi/ml; ICN), and 4 U of T4 polynucleotide kinase (Bethesda Research Laboratories) by incubation at 37°C for 30 min. The labelled primers were purified through a Sephadex G-50 column. One-tenth of the labelled primer and an appropriate amount of PCR product were used for each sequencing reaction. The synthesis reaction was carried out for 30 cycles of 30 s at 95°C and 60 s at 70°C according to the instructions provided by the manufacturer. The synthesized products were separated on 6 and 8% polyacrylamide denaturing wedge gels, and the gels were used to expose Kodak film overnight at room temperature without an intensifying screen. Each template was sequenced two or three times.

The sequences used for PCR amplification and double-stranded DNA cycle sequencing are as follows. The sequences for light-chain gene PCR amplification were VL (5′-GTGC CAGATGTGAGCTCGTGATGACCCAGTCTCCA-3′) and CL (5′TCCTTCTAGATTACTAACACTCTCCCCTGTTGA-3′). The sequences for light-chain variable-region gene sequencing were MK17 (5′-AGATGTTAACTGCTCAC-3′) and MK61 (5′-ACTGCCACTGAACCTG-3′). The sequences for heavy-chain gene PCR amplification were V_H [5′-AGGTC CAGCT(T/G)CTCGAGTCTGG-3′] for all isotypes, CH1 (5′-AGGCTTACTAGTACAATCCCTGGGCACAAT-3′) for IgG1, CH2 (5′-GATTGTACTAGTGGGCCCTCTGGGCTC-3′) for IgG2a/2b, CH3 (5′-AGGTGTACTAGTCTTGGG TATTCTAGG-3′) for IgG3 clones C1.1, -2, and -7, and CH3a (5′-TAGGGAGATCATGAGTG-3′) for IgG3 (clone C1.8). The sequences for heavy-chain variable-region gene sequencing were H125 (5′-GGCCAGTGGATAGAC-3′) for IgG1 and IgG2a/2b, H120 (5′-AGATGGGGCTGTTGTTG-3′) for IgG3, and H74 [5′-TGTT(C/G)(T/C)TGGCATT(G/C)TC-3′] for clones C1.1 to C1.6.

RESULTS

MAb production and epitope location. A total of 63 hybridomas secreting antibodies to serovar C EBs were raised in two immunized BALB/c mice, of which 44 (70%) recognized epitopes on MOMP by the immunoblot and/or radioimmunoprecipitation assay. Each MAb was reacted with a set of 370 complete overlapping hexapeptides covering the entire serovar C Omp1 sequence to localize their epitopes, and 12 epitopes were specifically mapped; 4 mapped to VD4 and 8 mapped to VD1. The eight MAbs whose epitopes mapped to VD1, designated C1.1 to C1.8 (Table 1), were selected for further study because each bound to the same nominal sequence (AGLQND) within VD1 of the serovar C MOMP; however, binding patterns to individual hexapeptides varied. From the binding pattern similarities, the eight MAbs were provisionally classified into two groups. MAbs C1.1 to C1.5 (designated group I) bound to three consecutive hexamers (AGLQND, GLQNDP, and LQNDPT) and thus required a minimum of four critical residues (LQND); MAbs C1.6 to C1.8 (group II) bound to a single hexamer with the highest binding activity and thus required a minimum of six residues (AGLQND). The eight MAbs exhibited different isotypes: C1.3, C1.5, and C1.6 were IgG1, C1.4 was IgG2b, and C1.1, C1.2, C1.7, and C1.8 were IgG3. All had kappa light chains.

Immunochemical characterization of MAb recognition of the epitope sequence. The MAb reactivities were compared with heat-treated or detergent-solubilized EBs in immunodot, radioimmunoprecipitation, and immunoblot assays (Fig. 1). All eight MAbs bound to both native and heat-treated EBs on dot blots. All eight MAbs precipitated MOMP from serovar C EBs treated with mild (0.5% desoxycholate, 0.1% sodium dodecyl sulfate [SDS], 0.5% Triton X-100) detergent. Only

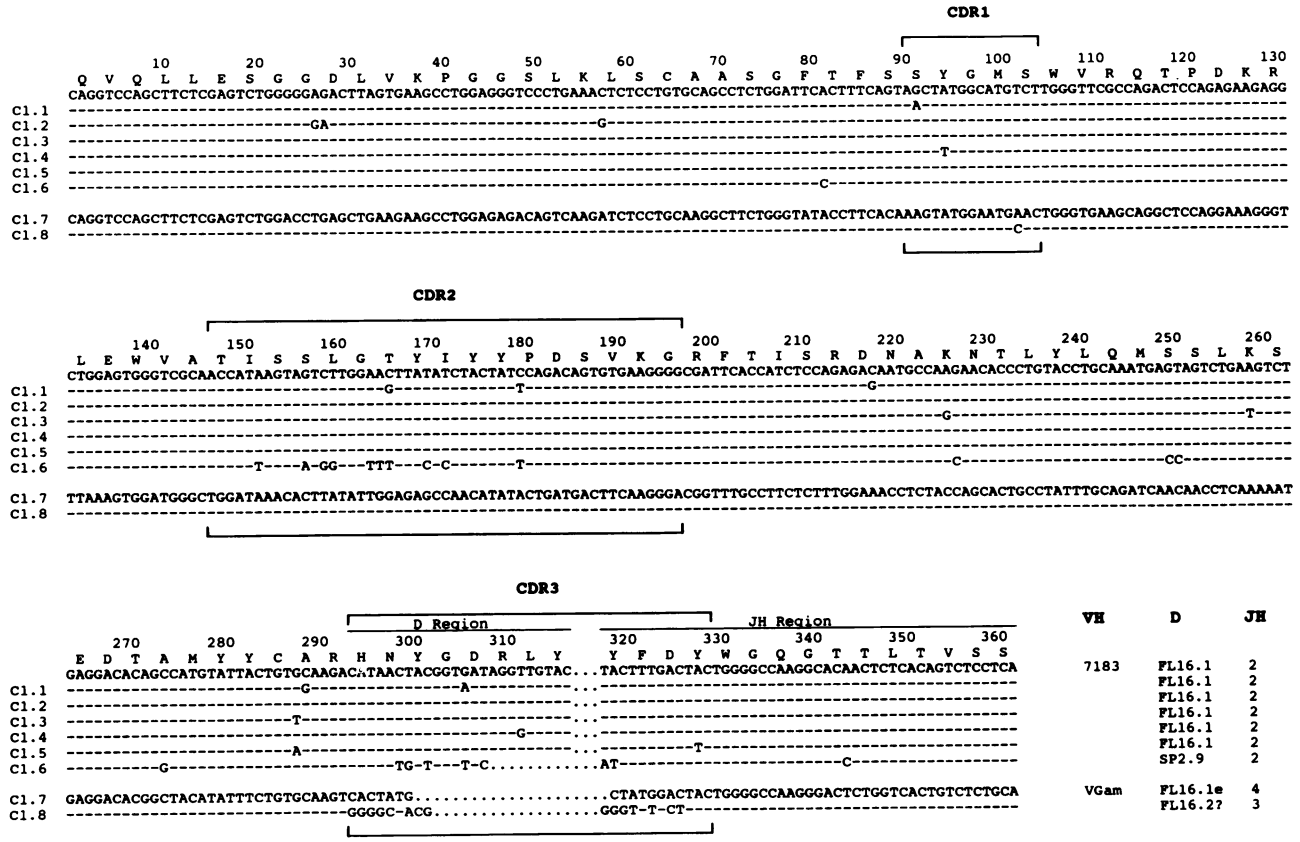


FIG. 4. Nucleotide sequences of the heavy-chain V regions. Sequences are grouped by V_H gene family and are displayed relative to a consensus sequence (for C1.1 to C1.6) generated by alignment of the sequences within the group or relative to each other (for C1.7 and C1.8). Dashes indicate identity with the consensus sequence (C1.1 to C1.6) or the top sequence (C1.7 and C1.8); differences are indicated. Gaps, represented by dots, have been introduced in CDR3 to facilitate alignment among the sequences. The nucleotide sequence data in this figure appear in the EMBL data base under accession numbers X67789 for C1.1H, X67799 for C1.2H, X67790 for C1.3H, X67791 for C1.4H, X67792 for C1.5H, X67793 for C1.6H, X67800 for C1.7H, and X67796 for C1.8H.

group I MAbs (C1.1 to C1.5) recognized SDS-denatured MOMP on immunoblot assays; group II MAbs (C1.6 to C1.8) did not bind MOMP under these conditions.

The binding of the eight MAbs with native EBs on a solid phase (standard ELISA) or in solution (competition ELISA) was next determined (Fig. 2). The eight MAbs were first titrated against native serovar C and A EBs coated on the plates in a standard ELISA (Fig. 2A and B). Serovar A was chosen for comparison with serovar C because it appears to be an antigenic-drift variant in this epitope region from serovar C. Both group I and II MAbs reacted with native serovar C EBs (Fig. 2A), but only MAb C1.6 reacted with serovar C and A EBs (Fig. 2D).

A competition ELISA was used to estimate the relative affinity of MAb binding to the native epitope in solution. All eight MAbs recognized the native epitope on the surface of serovar C EBs in solution, with the three group II MAbs exhibiting significantly higher affinity than the group I MAbs ($P < 0.001$) (Fig. 2B). Only MAb C1.6 had significant affinity for both serovar C and A EBs in the competition assay (Fig. 2E).

An in vitro neutralization assay was next used to evaluate the potential functional significance of the heterogeneity in MAb binding. Viable *C. trachomatis* serovar C (Fig. 2C) and serovar A (Fig. 2F) were used. All eight MAbs neutralized serovar C. Surprisingly, group II MAbs did not neutralize serovar C better than did group I MAbs. MAb C1.6 was able to neutralize both serovar C and A *C. trachomatis* in cell culture.

Determination of critical amino acids in the peptide epitope sequence. Having found that the MAbs differed in relative affinity and in serovar cross-reactivity, we next determined whether the pattern of "critical" amino acid recognition within the epitope sequence VAGLQND differed among the eight MAbs. Substitution analysis showed that not all residues in the minimal epitope, as determined by the use of overlapping hexapeptides, were equally important for antibody binding to the peptide epitope (Fig. 3). For clones C1.1 to C1.5, two nonconsecutive residues, L and N, interrupted by any other amino acid were most critical for the peptide epitope-antibody interactions. The critical residue pattern was expressed as L-X-N, where X represents the non-critical residue; similarly, the critical residue pattern was G-L-X-X-D for MAb C1.6, G-L-X-N-D for C1.7, and G-L-Q-N-D for C1.8.

These data provide a potential explanation for the ability of MAb C1.6 to recognize both serovars C and A whereas the other MAbs recognized only serovar C. Comparison of the serovar C peptide epitope sequence (VAGLQND) with that of the serovar A peptide epitope (VAGLEKD) shows that only MAb C1.6 has a critical amino acid recognition pattern (G-L-X-X-D) which would allow it to cross-react with serovar A.

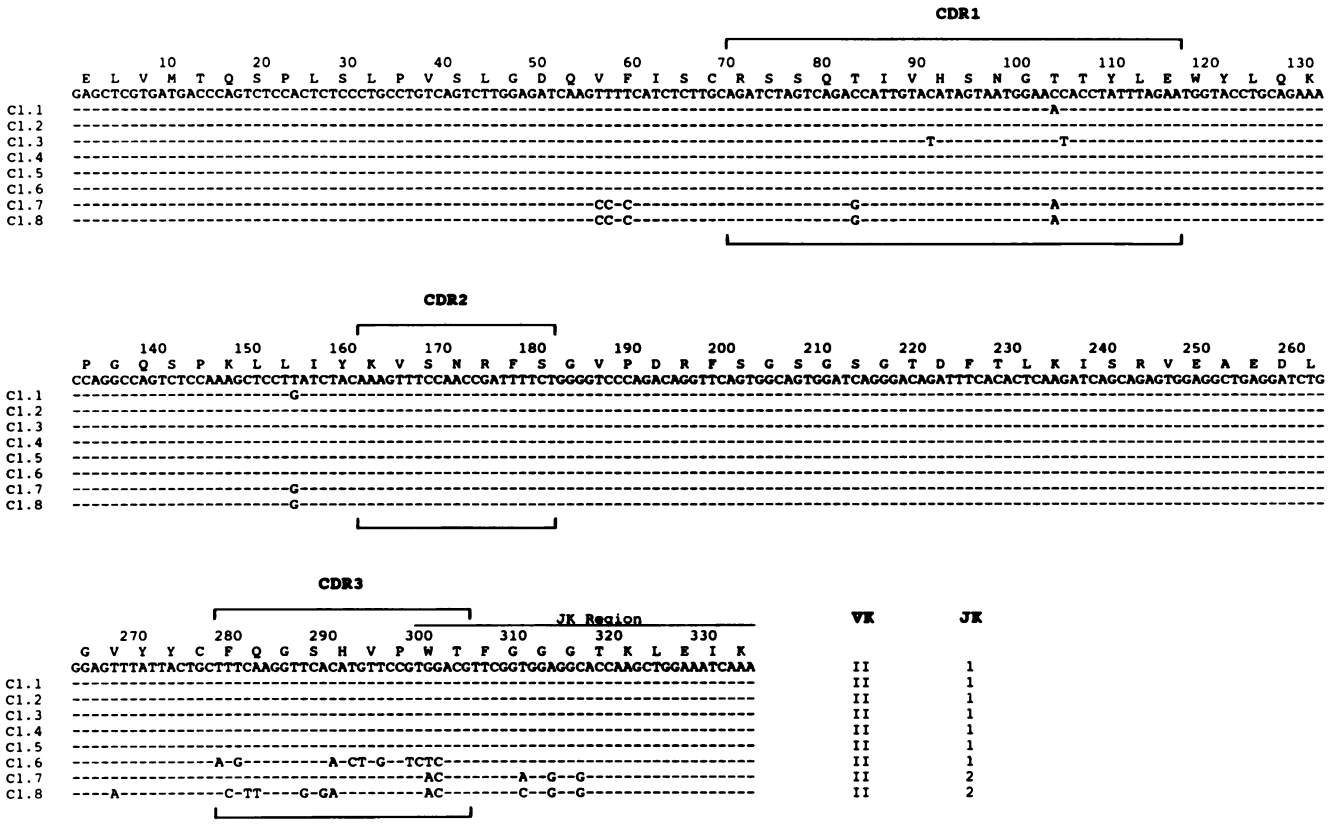


FIG. 5. Nucleotide sequences of the light-chain V regions. Sequences are displayed relative to a consensus sequence (the deduced amino acid sequence of the consensus sequence is also shown). Dashes indicate identity with the consensus sequence; differences are shown. The nucleotide sequence data in this figure appear in the EMBL data base under accession numbers X67794 for C1.1L, X67795 for C1.2L, X67801 for C1.3L, X67798 for C1.4L, X67797 for C1.5L, X67802 for C1.6L, X67803 for C1.7L, and X67804 for C1.8L.

DNA sequence analysis of the MAb variable-region genes. We next evaluated the genetic basis for the heterogeneity in affinity, fine specificity, and serovar cross-reactivity observed for these MAbs. The nucleotide sequence of the antibody variable (V) region was determined for both the heavy and light chains for each MAb. The nucleotide and deduced amino acid sequences of the heavy-chain V regions are shown in Fig. 4 (see also Fig. 6A). The V_H sequences were assigned to two V_H gene families on the basis of percent similarity to prototype sequences representative of 12 V_H gene families (10, 18, 20, 23). Antibodies C1.1 to C1.6 had V_H genes from the V_H7183 family, while MAb C1.7 and C1.8 had V_H genes from V_H-VGam family. Of the six MAbs (C1.1 to C1.6) that had V_H genes from the V_H7183 family, five (C1.1 to C1.5) had the same D segment from the DFL16.1 family and the same J segment from the J_H2 family. Thus, MAbs C1.1 to C1.5 had the same set, V_H7183-DFL16.1-J_H2, during V-region recombination. By comparing the sequences in clones C1.1 to C1.5 with the DFL16.1 germ line gene (TAT TAC TAC GGT AGT AGC) and J_H2 germ line gene sequences (20), it was evident that N sequences were generated at both the V_H to D border (AAC for all five clones; Fig. 4) and the D to J_H border (GAT AGG TTG TAC for C1.2, -3, and -5; AAT AGG TTG TAC for C1.1; and GAT AGG GTG TAC for C1.4) (Fig. 4). A two-codon deletion from both the 5′ end (TAT TAC) and 3′ end (AGT AGC) of the DFL16.1 germ line gene also occurred during the V_H-D-J_H recombination for these five MAbs.

C1.6 had the same V_H gene as C1.1 to C1.5 (V_H7183) but a D segment from the DSP2.9 family, which resulted in a different CDR3 sequence. In addition, C1.6 exhibited not only a different D gene sequence but also a three-amino-acid deletion in the CDR3 region. Presumably, this occurred during recombination.

C1.7 and C1.8 had V_H genes from the VGam family but distinctive D and J minigenes: DFL16.1e and J_H3 for C1.7 and DFL61.2 and V_H4 for C1.8. The lengths of the CDR3 regions differed among C1.1 to C1.6, which suggests that different joining events occurred during gene assembly.

The nucleotide and deduced amino acid sequences of the light-chain V regions of the eight MAbs are shown in Fig. 5 and 6B). All eight MAbs had V_κ genes from the same V_κII family. Since the myeloma cell line (NS1) used in this study can synthesize its own kappa chain, we searched for homology between the V_κ genes from the eight hybridomas and the NS1 kappa gene, but none was found. C1.1 to C1.6 had the same J_κ1 segment; C1.7 and C1.8 had a different J_κ2 segment. No de novo sequences or deletions occurred in CDR3 regions during V_κ-J_κ recombination, since the CDR3 regions were identical in length.

DISCUSSION

Antibody responses to *C. trachomatis* appear to be highly focused on discrete segmental regions (the VDs) of the MOMP (21). This may be due to the tertiary structural arrangement of the protein in the outer membrane of the

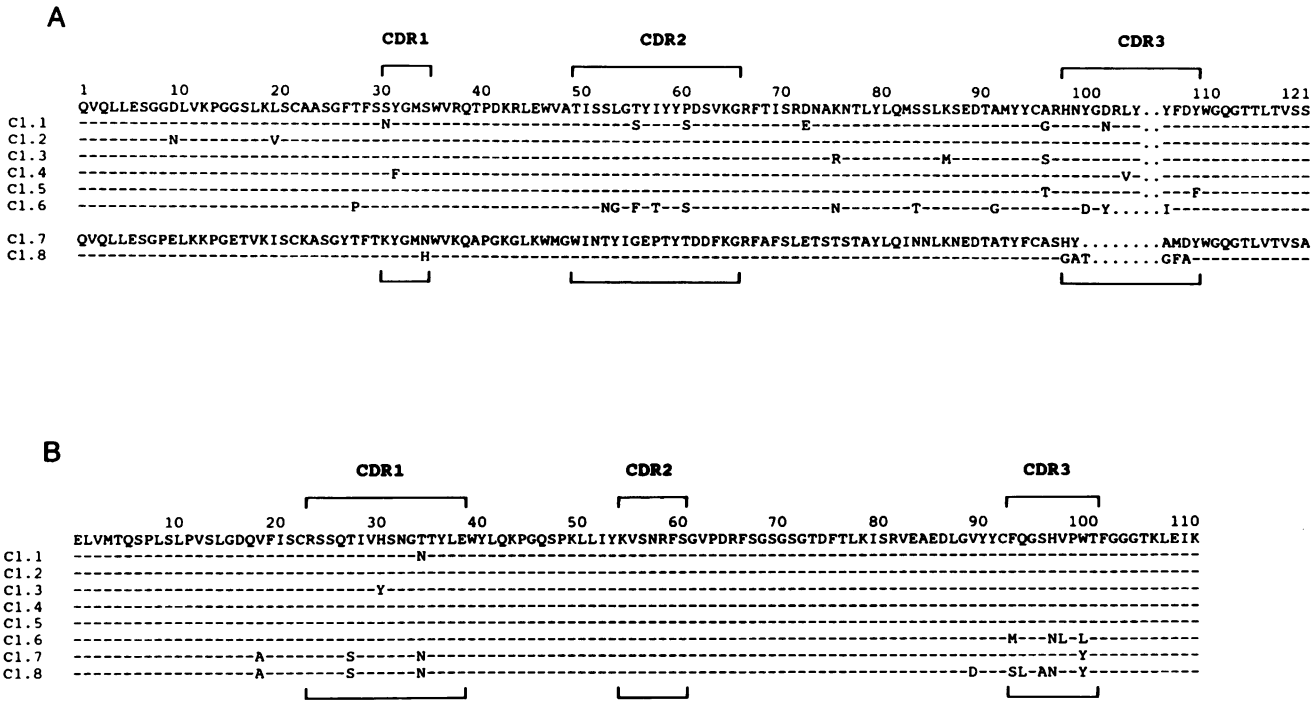


FIG. 6. Deduced amino acid sequences of heavy-chain (A) and light-chain (B) V regions. Sequences are grouped according to V_H or V_κ family and displayed in reference to their corresponding consensus sequences. Identity with the consensus (top) sequence is indicated by dashes; differences are shown. Dots indicate gaps introduced to facilitate alignment.

bacteria, with the immunogenic regions arrayed as surface-exposed loops (2). The data presented in this article suggest that multiple B-cell clones are involved in the recognition of a single epitope on a chlamydial MOMP VD, and we speculate that such pleiotropic antibody recognition has functional significance by affecting the rate at which the immune system can select antigenic escape variants through single-site mutations in a MOMP VD.

Eight distinct MAbs that recognized the same neutralizing epitope sequence on the MOMP were produced in two BALB/c mice after immunization with *C. trachomatis* serovar C EBs. The epitope recognized by each of the eight MAbs was located in VD1 of serovar C. Although each MAb recognized a common heat-stable epitope (VAGLQND) sequence, the MAbs each had a characteristic fine-specificity pattern of amino acid recognition and distinct affinity and were classifiable into two groups. Group I, composed of MAbs C1.1 to C1.5, recognized a tetramer peptide (LQND) and recognized MOMP in both radioimmunoprecipitation and immunoblot assays. Group II, composed of MAbs C1.6 to C1.8, recognized a longer peptide epitope (AGLQND) and bound MOMP only in the radioimmunoprecipitation and not in the immunoblot assay. Group II MAbs bound the epitope on native EBs with about a sevenfold-higher affinity than group I MAbs. Both group I and group II MAbs effectively neutralized serovar C in cell culture. These data suggest that the immunochemistry of the epitope-paratope interaction is distinct for the group I and group II MAbs.

To further probe the immunochemistry of antibody recognition of the peptide epitope, we characterized the ability of each MAb to bind to serially substituted peptide analogs of the parent sequence and to cross-react with a serologically closely related *C. trachomatis* serovar, serovar A. This serovar has a modified serovar C sequence in VD1 (from VDGLQND to VAGLGKD).

Amino acid substitution analysis showed that each MAb had a reproducible fine-specificity pattern of epitope recognition. The pattern for MAbs C1.1 to C1.5 was -L-X-N-, that for C1.6 was -G-L-X-X-D, that for C1.7 was -G-L-X-N-D, and that for C1.8 was -G-L-Q-N-D-. From the pattern of critical amino acids required for recognition, only MAb C1.6 would be predicted to bind and neutralize serovar A as well as serovar C; this prediction was confirmed. The results suggest that critical residues found by the pepscan approach are also functionally critical in antibody-mediated neutralization.

Genetic analysis of antibody genes from each of the eight hybridomas showed that the immunochemical differences for group I and group II MAb binding to the peptide epitope were related to different V_H genes and junctional diversity. The differences in the fine specificity of amino acid recognition by each MAb were correlated with antibody V-region sequence variability. Overall, the eight MAbs had heavy chains encoded by two different V_H gene families, two different D_H genes, and three different J_H genes. The light chains were less diverse than the heavy chains, with a single V_κ gene and two different J_κ genes.

B-cell clones recognizing the epitope sequence in the group I pattern (clones C1.1 to C1.5) had closely related antibody genes. B-cell clones recognizing the epitope sequence in a group II pattern had different gene fragments. The fine specificity of epitope recognition for C1.1 to C1.5 (group I) was very similar: all required L and N as critical amino acid residues (critical residue pattern, -L-X-N-), whereas MAb C1.6 (group II) displayed a distinct fine-specificity pattern, with residues G, L, and D being nonreplaceable (-G-L-X-X-D-). Although all six of the MAbs had identical V_H genes, the

difference in fine specificity of recognition was attributed to the use of a distinct D minigene, DSP.2 in MAb C1.6 and DFL16.1 in MAb C1.1 to C1.5. The fine-specificity patterns of C1.7 and C1.8 were different from those of the other six MAbs. C1.7 required residues G-L and N-D but not Q, and C1.8 required all five consecutive residues G-L-Q-N-D. Presumably, the presence of a V_H gene from a distinct V_H family (VGam) determined this shift in fine specificity. The fine-specificity difference between MAbs C1.7 and C1.8 probably arises from the use of different D and J_H segments (DFL16.1e and J_H4 for C1.7 versus DF16.2 and J_H3 for C1.8). In aggregate, these results suggest that amino acid residues that are found to be critical for antibody binding in the pepscan assay are reflected in the use of different V-region genes by different hybridomas.

The genetic heterogeneity of antibody specificity and the immunochemistry of antigen recognition that we have defined for MAbs to a MOMP epitope may be functionally significant. The epitope that we have characterized is antigenically variable among related chlamydial serovars and apparently has undergone antigenic drift (16). Since each MAb was capable of neutralizing the organism and required distinct critical amino acids in the epitope for antibody binding, antigenic variation by point mutation within the epitope sequence could allow the organism to escape neutralization by antibodies of one specificity class. For instance, residue N in epitope sequence VAGLQND from serovar C, when mutated to K in the corresponding epitope sequence of serovar A, allows serovar A to escape neutralization by some of the MAbs that we studied. However, a polyclonal antibody response that displays distinct patterns of epitope recognition is characteristic of the intact animal. For instance, MAbs C1.1 to C1.5, C1.7, and C1.8 were all isolated from a single mouse. Thus, the host, by generating antibodies of differing fine specificities through the use of multiple V, D, and J region minigenes and perhaps through the process of somatic hypermutation, may minimize the chance for a pathogen to develop antigenic escape variants through point mutation (16, 19). It will be important to determine whether oligopeptide vaccination also induces a pleiotropic B-cell response.

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