

Detection of *Chlamydia pneumoniae*-Specific Antibodies Binding to the VD2 and VD3 Regions of the Major Outer Membrane Protein

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Although *Chlamydia pneumoniae* is an important human pathogen, the antigens eliciting a specific humoral immune response remain elusive. We scrutinized several recombinant chlamydial surface proteins for species-specific recognition by a panel of human sera previously tested for the presence of anti-*C. pneumoniae* and anti-*C. trachomatis* antibodies by microimmunofluorescence and enzyme-linked immunosorbent assay. The 15-kDa cysteine-rich protein (CrpA), porin-b (PorB), 9-kDa outer membrane protein (OMP3), 60-kDa outer membrane protein (OMP2), and four fragments of the major outer membrane protein (MOMP) representing each variable domain (VD) were overexpressed in *Escherichia coli*, affinity purified, and employed for Western blot analysis. None of the sera tested contained antibodies recognizing PorB and OMP3 of *C. pneumoniae*. Sera from *C. pneumoniae*-immune patients cross-reacted with OMP2 of *C. trachomatis*, and sera from *C. trachomatis*-immune patients cross-reacted with CrpA of *C. pneumoniae*, indicating that some of chlamydial surface molecules share antigenic epitopes. In contrast, the VD2, as well as the VD3, regions of the MOMP of *C. pneumoniae* were only recognized by *C. pneumoniae*-positive sera, suggesting the existence of species-specific epitopes. The identification of such epitopes of cell surface molecules provides new insights into *C. pneumoniae*-specific immune responses and may be of value for the improvement of *C. pneumoniae*-specific diagnostic assay systems based on defined recombinant antigens.

The obligate intracellular bacterium *Chlamydia pneumoniae* has been recognized as an important cause of several respiratory diseases in humans that gives rise to an average of 10% of cases of community-acquired pneumonia (17). Infection with *C. pneumoniae* has been implicated in the pathogenesis of severe cardiovascular diseases, especially atherosclerosis (32); however, the role of this pathogen as etiologic agent has not yet been proven and is still controversial (4, 13, 23, 31). Likewise, the possible involvement of *C. pneumoniae* in the pathogenesis of neurodegenerative diseases and lung cancer is intensely debated (18, 43). Antibodies to this organism are spread worldwide in the adult population (17). Analyses of various independent *C. pneumoniae* isolates revealed a high level of sequence homology (14, 29, 33). In contrast to *C. trachomatis*, for which 19 different serovars exist (20), only a few differences between different *C. pneumoniae* strains on a genetic, pathogenic, or serologic level have been revealed (9, 12, 17, 36). Although infections with *C. pneumoniae* can be monitored by direct isolation of the agent or by PCR-based detection of species-specific nucleotide sequences, serology represents the current routine method for a fast and convenient diagnosis. The microimmunofluorescence (MIF) assay, originally developed for *C. trachomatis* (38), is being considered as a sensitive and specific serologic method for the detection of *C. pneumoniae* infections (37). However, the diagnostic value of the results of the MIF test strongly depends on

the antigen preparation and the experience of the individual investigator (7, 25). Partially automated enzyme-linked immunosorbent assays (ELISAs) have also been developed for routine chlamydial diagnostics (10, 27). Both MIF and ELISAs are based on preparations of entire elementary bodies, which explains their inherent problems concerning cross-reactivities between *Chlamydia* species and even unrelated microorganisms (10, 15, 24, 34).

Parts of the chlamydial outer membrane complex and especially the major outer membrane protein (MOMP) have long been considered as primary targets of the humoral immune response in humans (5, 17). Despite extensive studies on the antigenic composition of *C. pneumoniae*, the existence of species-specific and/or immunodominant surface antigens is still puzzling. The MOMP of *C. pneumoniae* has been described as an immunodominant antigen (11, 12, 28). Most of the immunogenic epitopes of MOMP appear to be conformation dependent because monoclonal antibodies raised against denatured MOMP display low binding affinity (8, 40). MOMP is considered a porin, spanning the outer chlamydial membrane (30, 41). Sequence comparisons of the MOMPs from several *Chlamydia* species revealed the existence of four variable domains (VD1 to VD4), presumably surface exposed, flanked by five strictly conserved regions (26, 42). The murine antibody response to peptides representing the VDs of *C. pneumoniae* MOMP has been previously characterized (28, 40). However, the individual humoral immune response to each of these VDs in humans has not yet been resolved.

Besides MOMP, other members of the outer membrane complex have been described in *Chlamydia* as being immunogenic, e.g., porin-b or the cysteine-rich outer membrane proteins CrpA, OMP2, and OMP3 (16, 19, 22, 39, 44). However, only little information is available about species-specific anti-

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TABLE 1. Oligonucleotides used to generate recombinant chlamydial fusion proteins

Organism and protein	Oligonucleotide pairs (5'→3') ^a	Expression vector, cloning site(s)
<i>C. pneumoniae</i>		
CrpA	GAGctcgag ATGTCATCAAATCTACATCC GATctcgag CTAAACGCGAGCTATTTTACT	pET-14b, <i>Xho</i> I
PorB	TAggatcc CTTGACATGAATAGCAAGATG TTggatcc TCTAAACTGCAGACCTGACGT	pET-14b, <i>Bam</i> HI
OMP2	AGctcgag ATGTCCAAACTCATCAGACGAG CTTctcgag TTAATACACGTGGGTATTTTC	pET-14b, <i>Xho</i> I
OMP3	AAActcgag AAGAAAGCTGTTTAATGCT CCActcgag TTACTGTTTGCATCTGCCATC	pET-14b, <i>Xho</i> I
MOMP-VD1	GTGCGACGgaattc GCTTAGCTGCTGG AGCTCCTgaattc CAGAAAACATCAAAGCG	pGEX-2T, <i>Eco</i> RI
MOMP-VD2	CTTTGATggatcc TGTACTTTAGGAGC AACTTTAGGTTTggatcc TGCATATTGG	pGEX-2T, <i>Bam</i> HI
MOMP-VD3	CTTATGggatcc GGTTGTGCAACTTTGG GGATGTTATCaggatcc AAAGTTGCTCG	pGEX-2T, <i>Bam</i> HI
MOMP-VD4	CCATACATTGgaattc CAATGGTCTCGA GAATCTgaattc ACCAGATACGTGAGC	pGEX-2T, <i>Eco</i> RI
<i>C. trachomatis</i>		
CrpA	AGGAATGTAActcgag AGCACTGTACCCGT TAGAGCAATctcgag TCATTGGGTCTGATCC	pET-14b, <i>Xho</i> I
OMP2	AAAATAActcgag CGAATAGGAGATCCTATG CATCGATAAAAActcgag ATTAATAGATGTGTG	pET-14b, <i>Xho</i> I
MOMP-VD2	ATCGTTTIGATggatcc TGTACATTAGGAG TAGGTTTgaattc AGCATATTGGAATGAAGC	pGEX-2T, <i>Bam</i> HI/ <i>Eco</i> RI
MOMP-VD3	CTTTGTGGgatcc GGATGTGCAACTTTAGG TCGTATCgaattc AAAGCTTGCTCGAGACC	pGEX-2T, <i>Bam</i> HI/ <i>Eco</i> RI
MOMP-VD4	AGTTAAAggatcc CGAGCAAGCTTTGATGC TTAGAAGCGgaattc TGCATTACGTGAGC	pGEX-2T, <i>Bam</i> HI/ <i>Eco</i> RI

^a Inserted restriction sites are indicated in lowercase letters. Chlamydial gene sequences (in boldface) are derived from *C. pneumoniae* CWL-029 and *C. trachomatis* serovar D, respectively.

bodies reacting with the corresponding surface antigens of *C. pneumoniae*.

In the present study we performed a comparative serologic analysis of several prominent surface antigens of *C. pneumoniae* and *C. trachomatis* in immunoblot assays with particular emphasis on their MOMP. Since these proteins possess an obviously polyantigenic character, fragments of MOMP were expressed as recombinant fusion proteins. The immunoreactivity of these fragments was analyzed by using a panel of human sera that were previously tested for anti-*C. pneumoniae* and anti-*C. trachomatis* antibodies.

MATERIALS AND METHODS

Chlamydial strains and genomic DNA. *C. pneumoniae* strain CWL-029 and *C. pneumoniae* isolates were obtained from M. Maass, University of Lübeck, Lübeck, Germany. Genomic DNA from *C. trachomatis* serovar D was provided by E. Straube, University of Jena, Jena, Germany.

Serum samples. Human sera were collected from independent clinic patients seropositive for *C. pneumoniae* and/or *C. trachomatis*, as well as from apparently healthy donors.

MIF and ELISAs. The levels of immunoglobulins (immunoglobulin G [IgG] and IgA) against *C. pneumoniae* and *C. trachomatis* in serum were determined semiquantitatively with SeroCP/CT ELISA kits (Hain Diagnostika, Nehren, Germany) according to the manufacturer's instructions, respectively. Optical densities at 450 nm of ≥ 1.4 for *C. pneumoniae* and ≥ 1.2 for *C. trachomatis* were considered positive. Additionally, detection of specific IgG was carried out by MIF assay with a *C. pneumoniae* MIF kit (Virion, Munich, Germany). Serum titers of $\geq 1:20$ were considered positive.

Molecular cloning of *C. pneumoniae* and *C. trachomatis* genes. *C. pneumoniae* was propagated in HEP-2 cells (35) and elementary bodies were isolated (21). Bacterial DNA was purified by using a DNeasy DNA extraction kit (Qiagen). Genes and gene fragments coding for CrpA, PorB, OMP2, OMP3, and the separate variable MOMP domains VD1 to VD4 were amplified by PCR by using *Pfu* polymerase (Stratagene) and oligonucleotide primers flanked with specific restriction sites (Table 1 and Fig. 1). The primer design was based on chlamydial sequences obtained from published data banks (14). After restriction of the PCR products with the indicated restriction enzymes, DNA fragments were cloned

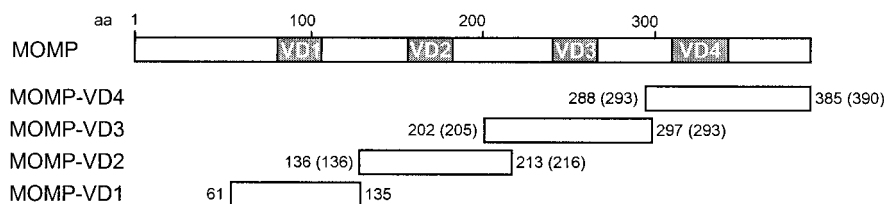


FIG. 1. Molecular cloning of separate VDs of the *C. pneumoniae* and *C. trachomatis* MOMP. The positions of the VDs are depicted schematically within the chlamydial full-length MOMP. The coding regions of the indicated MOMP fragments VD1 to VD4, along with flanking sequences, were individually cloned into a pGEX-2T expression vector, respectively (see Table 1). Amino acid (aa) positions are indicated for *C. pneumoniae* and *C. trachomatis* (in parentheses).

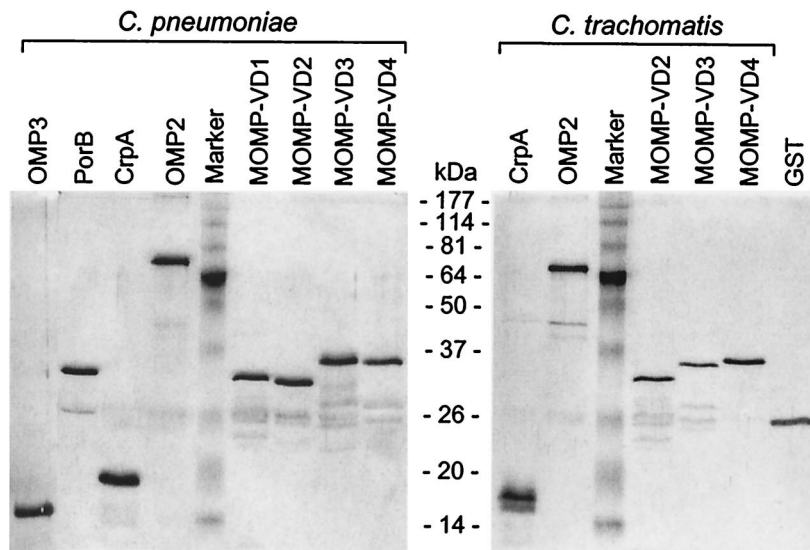


FIG. 2. Purified recombinant fusion proteins of *C. pneumoniae* and *C. trachomatis*. The indicated protein preparations were separated by SDS-PAGE, followed by silver staining.

into the protein expression vectors pET-14b (Novagen) and pGEX-2T (Pharmacia), respectively (Table 1). The identity of clones was confirmed by nucleotide sequencing in both directions over the entire DNA insert.

Expression and purification of recombinant fusion proteins. As indicated in Table 1, chlamydial genes were overexpressed in *Escherichia coli* BL21(DE3) as proteins fused to either glutathione *S*-transferase (GST) or an N-terminal hexahistidine tag (His₆). Bacteria were grown at 37°C in 2×YT medium (Difco) containing 200 µg of ampicillin/ml and 1% glucose with constant shaking at 225 min⁻¹. Overexpression of fusion proteins was induced at an optical density at 600 nm of 0.5 to 1.0 by the addition of 0.4 mM (for His₆ proteins) or 0.25 mM (for GST proteins) IPTG (isopropyl-β-D-thiogalactopyranoside). After a 2-h incubation at 30°C, cells were harvested and lysed by three consecutive freeze-thaw cycles in the presence of lysis buffer (for His₆ proteins, 20 mM Tris-HCl [pH 8.0], 0.5 M NaCl, 0.2% Triton X-100, 2 mM 2-mercaptoethanol, 8 M urea; for GST proteins, 20 mM HEPES-KOH [5], 140 mM NaCl, 1% Triton X-100, 2 mM 2-mercaptoethanol) and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 µg of pepstatin A/ml, 1 µg of leupeptin/ml), followed by short ultrasonication. After centrifugation (20,000 × g, 30 min), supernatants were subjected to affinity chromatography by using glutathione-Sepharose 4B or Chelating Sepharose Fast Flow (both from Pharmacia) according to the manufacturer's protocols, respectively. GST fusion proteins were eluted by adding 10 mM reduced glutathione and His-fused proteins by successively increasing the imidazole concentration to 0.5 M. The quality of the recovered protein fractions was judged after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining (1). Protein concentrations were determined with a colorimetric BCA kit (Pierce). Finally, in-frame expression of the proteins was confirmed by mass spectrometry as previously described (3).

Immunoblot analysis. The reactions of recombinant fusion proteins with human serum immunoglobulins were analyzed by Western blot assays according to standard protocols (1). In brief, protein preparations were subjected to a SDS-13% PAGE. The gels were then electroblotted onto a Hybond C nitrocellulose membrane (Amersham). Proper expression and size of the fusion proteins was confirmed by using a monoclonal anti-His₆ antibody (dilution, 1:3,000; Qiagen) or goat anti-GST antiserum (dilution, 1:1,000; Pharmacia), followed by the horseradish peroxidase-conjugated secondary antibodies goat anti-mouse IgG and rabbit anti-goat IgG (both from Sigma at dilutions of 1:3,000 each), respectively.

The detection of interactions between antibodies of each MIF- or ELISA-tested serum sample and the recombinant chlamydial fusion proteins was performed with ca. 0.1 µg of purified protein per lane and serum dilutions of 1:200. Human immunoglobulins (IgG and IgA) were detected with horseradish peroxidase-conjugated secondary antibodies from Dako (Glostrup, Denmark) used at a 1:1,000 dilution. Signals were detected by enhanced chemiluminescence (Amersham).

RESULTS AND DISCUSSION

Selection of human sera by ELISA and MIF. IgG and IgA titers to both *C. pneumoniae* and *C. trachomatis* were detected by ELISA. In addition, the sera were subjected to a IgG MIF test. When the results of both ELISA and MIF were consistent, the sera were chosen for further experiments. Four serum groups were distinguished: group I (Cp⁺/Ct⁺) represents sera positive for both *C. pneumoniae* and *C. trachomatis* (*n* = 8); group II (Cp⁺/Ct⁻) represents sera only positive for *C. pneumoniae* (*n* = 20); group III (Cp⁻/Ct⁺) represents sera only positive for *C. trachomatis* (*n* = 6); group IV (Cp⁻/Ct⁻) represents sera that tested negative for chlamydial antibodies (*n* = 7).

Western blot analysis of recombinant chlamydial proteins. All chlamydial fusion proteins were obtained with a correct molecular weight and high purity (Fig. 2). Additional faint bands of lower molecular size were also visible in some of our protein preparations. They were identified as cleavage products of the fusion proteins in Western blots with anti-GST antiserum (data not shown).

In order to evaluate the immunoreactivity of the purified chlamydial surface antigens, each of the 41 sera belonging to one of the four different serum groups I to IV was analyzed by Western blotting. A reaction pattern of representative sera of each serogroup is shown in Fig. 3. The results obtained by Western blot analysis of all sera are summarized in Fig. 4. Porin-b (PorB) and the 9-kDa outer-membrane protein (OMP3) of *C. pneumoniae* were not recognized by IgG or IgA antibodies in any of the human sera tested (Fig. 4). Whether PorB and OMP3 are poorly immunogenic or whether putative linear or conformation-dependent epitopes were lost due to denaturing Western blot conditions remains unclear.

As shown in Fig. 3A to C and 4A to C, the strongest and most frequent reactions were observed toward the recombinant OMP2 proteins of both *C. pneumoniae* and *C. trachoma-*

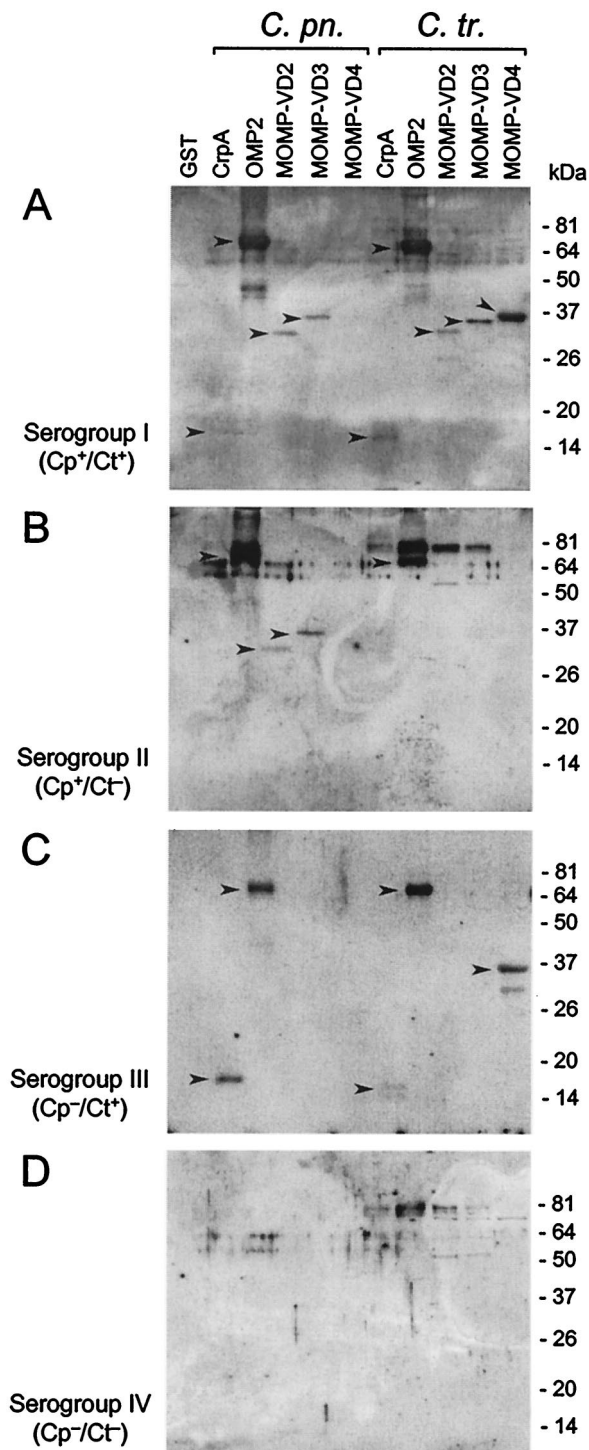


FIG. 3. Western blot analysis of recombinant surface proteins of *C. pneumoniae* (*C. pn.*) and *C. trachomatis* (*C. tr.*) with human sera previously tested for the presence of *Chlamydia*-specific antibodies. IgG reactivity is shown for representative serum samples corresponding to four groups. (A) Group I, seropositive for both *C. pneumoniae* and *C. trachomatis*. (B) Group II, seropositive for *C. pneumoniae* only. (C) Group III, seropositive for *C. trachomatis* only. (D) Group IV, seronegative for both *C. pneumoniae* and *C. trachomatis*. *Chlamydia*-specific reactions are highlighted by arrowheads.

tis. Notably, all sera that tested positive only for *C. pneumoniae* reacted with OMP2 from both *C. pneumoniae* and *C. trachomatis* (Fig. 4B). Likewise, *C. trachomatis* immune sera displayed OMP2 reactivity at considerably less frequency but with a significant degree of cross-reactivity to OMP2 of *C. pneumoniae* (Fig. 3C and 4C). This indicates that the OMP2 proteins from *C. pneumoniae* and *C. trachomatis* share immunodominant epitope(s). Indeed, the primary structure of both polypeptides is highly conserved (14, 22). The question arose as to why this strong cross-reaction of *C. pneumoniae* immune sera with OMP2 of *C. trachomatis* does not yield positive results in ELISA or MIF assay for *C. trachomatis*. It has been shown that the chlamydial cysteine-rich 60-kDa proteins are scarcely accessible, if at all, for antibody binding at the surface of intact bacteria or when whole elementary bodies were presented (22, 39). However, denatured and truncated chlamydial OMP2 fusion proteins have been reported to be major immunogens in chlamydial infections in a genus-specific manner (6, 22, 39). As a result of host defense reactions during the course of infection, determinants of previously inaccessible bacterial proteins may be released from disintegrated cell walls and become exposed to B-cell antigen receptors. Furthermore, upon protein unfolding after limited proteolysis, previously inaccessible linear motifs can be exposed to serve as B-cell epitopes.

Antibody binding to the recombinant 15-kDa cysteine-rich proteins (CrpA) of both *Chlamydia* species was mainly observed with sera belonging to groups I, II, and III (Fig. 3 and 4). The apparent cross-reactivities can be explained by a strong sequence homology (38%) between CrpA of *C. pneumoniae* and *C. trachomatis* (14). However, sera from the uninfected group IV also contained antibodies to CrpA of both *Chlamydia* species (Fig. 4D). These observations are consistent with previous reports that showed weak or nonspecific reactivities with chlamydial 15-kDa outer membrane proteins (5, 8). Seroreactivity with the proteins CrpA, OMP2, and MOMP-VD4 was also shown for one of the seven sera, which were previously classified into group IV (Cp⁻/Ct⁻) (Fig. 4D). However, since the reliability and specificity of commercial MIF assay and ELISA is limited (10, 24, 34), this serum may have tested false negative for the presence of chlamydial antibodies.

The immunoreactivity against the chlamydial MOMP was investigated by using defined protein fragments representing VDs 1 to 4 (MOMP-VD1 to MOMP-VD4) (Fig. 1). Since the immunoblot analysis of the different MOMP fragments was performed with proteins fused to GST, GST polypeptide were not observed with any of the sera under investigation, indicating that the immunoreactivities detected can be sensed to be *Chlamydia* specific (Fig. 3). Although fragment MOMP-VD1 from *C. pneumoniae* contains a predicted extracellular VD, it showed no reactivity with IgG or IgA from human sera in Western blots (data not shown). As shown in Fig. 3A and B and 4A and B, the MOMP-VD2 and MOMP-VD3 fragments of *C. pneumoniae* were only recognized by sera that tested positive in a MIF assay and an ELISA specific for *C. pneumoniae*. No significant immunoreactivity to this domain was observed when *C. pneumoniae*-negative sera from group III (Cp⁻/Ct⁺, Fig. 3C and 4C) or group IV (Cp⁻/Ct⁻, Fig. 3C and 4C) were used. Correspondingly, the MOMP VD3 region of *C. trachomatis* was only recognized by *C. trachomatis* positive

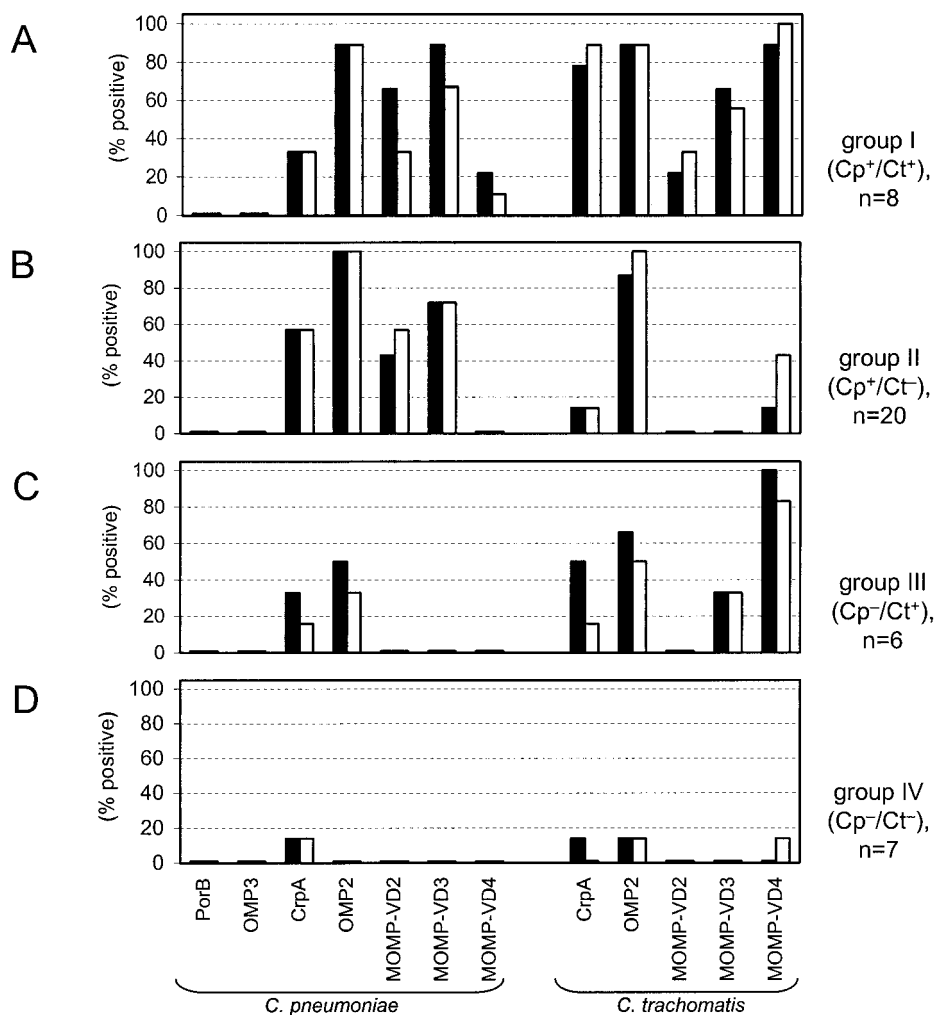


FIG. 4. Immunoreactivity of serum antibodies with recombinant chlamydial surface proteins. Immunoglobulins (IgA and IgG) recognizing the indicated fusion proteins of *C. pneumoniae* and *C. trachomatis* were detected by Western blotting in a cohort of 41 human sera belonging to serogroups I to IV (panels A to D, respectively). The frequencies of positive reactions are presented for each serogroup and each individual chlamydial antigen. Secondary antibodies were directed against IgG (solid bars) and IgA (open bars).

sera (Fig. 4C). Notably, IgA immunoreactivity patterns were similar to those of IgG with regard to species-specific recognition of VD2 and VD3. Since at least 19 serovars of *C. trachomatis* exist, the possibility remains that antibodies to the VD2/VD3 regions of MOMP of a specific *C. trachomatis* serovar not represented in the patient group III might cross-react with the corresponding regions of *C. pneumoniae* MOMP.

Reactions with the MOMP VD4 region of *C. pneumoniae* were scarce (Fig. 4). In contrast, MOMP-VD4 of *C. trachomatis* yielded strong signals when *C. trachomatis*-positive sera were tested (Fig. 3A and C and 4A and C). This finding is consistent with a previous report wherein species- and serovar-specific epitopes were mapped to the VD4 region of the *C. trachomatis* MOMP by using murine monoclonal antibodies and different synthetic peptides (2). Some additional bands were visible in a size range between 50 and 75 kDa, in particular when *C. trachomatis* protein preparations were used (Fig. 3). These were probably due to interactions of human antibody

with *E. coli* proteins contaminating our preparations of recombinant chlamydial proteins.

We have confirmed and extended previous reports describing the immunogenicity of surface-exposed chlamydial proteins. Up to the present, it has been difficult to show species-specific immunoreactivity of surface antigens of *C. pneumoniae*. This held especially true for the MOMP of *C. pneumoniae*, which is an immunodominant and functionally important protein within the chlamydial infection cycle (11, 12, 36). We show here that it is possible to avoid frequently reported cross-reactivities of antibodies when the MOMP protein is dissected into fragments according to its extracellular VDs. Although VD4 may allow for the detection of *C. trachomatis*-specific antibodies, the identification of VD2 and VD3 regions of *C. pneumoniae* as species-specific domains within the MOMP may prove valuable for the improvement of species-specific assay systems for *C. pneumoniae*. Furthermore, the identification of species-specific recognition of well-defined proteins should allow new insights into humoral immune responses against *C. pneumoniae* and *C. trachomatis*.

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