

Antigenic Topology of Chlamydial PorB Protein and Identification of Targets for Immune Neutralization of Infectivity¹

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The outer membrane protein PorB is a conserved chlamydial protein that functions as a porin and is capable of eliciting neutralizing Abs. A topological antigenic map was developed using overlapping synthetic peptides representing the *Chlamydia trachomatis* PorB sequence and polyclonal immune sera. To identify which antigenic determinants were surface accessible, mono-specific antisera were raised to the PorB peptides and were used in dot-blot and ELISA-based absorption studies with viable chlamydial elementary bodies (EBs). The ability of the surface-accessible antigenic determinants to direct neutralizing Ab responses was investigated using standardized in vitro neutralization assays. Four major antigenic clusters corresponding to Phe³⁴-Leu⁵⁹ (B1-2 and B1-3), Asp¹¹²-Glu¹⁴⁵ (B2-3 and B2-4), Gly¹⁷⁹-Ala²²⁵ (B3-2 to B3-4), and Val²⁶¹-Asn³⁰⁵ (B4-4 to B5-2) were identified. Collectively, the EB absorption and dot-blot assays established that the immunoreactive PorB Ags were exposed on the surface of chlamydial EBs. Peptide-specific antisera raised to the surface-accessible Ags neutralized chlamydial infectivity and demonstrated cross-reactivity to synthetic peptides representing analogous *C. pneumoniae* PorB sequences. Furthermore, neutralization of chlamydial infectivity by *C. trachomatis* PorB antisera was inhibited by synthetic peptides representing the surface-exposed PorB antigenic determinants. These findings demonstrate that PorB Ags may be useful for development of chlamydial vaccines. *The Journal of Immunology*, 2002, 168: 5184–5191.

Complications of human chlamydial infections remain a major public health concern and account for significant morbidity worldwide (1). In regions where poor economic conditions predominate, young children have a high rate of ocular exposure to *Chlamydia trachomatis* and comprise the reservoir of the organism (2). Persistent and recurrent chlamydial infection in these children leads to severely scarred conjunctivae and subsequent blindness as the population ages (2). Genital chlamydial infections present unique problems because the majority of infections in women are asymptomatic. Unrecognized and untreated, the bacteria persist in the host as chronic infections, posing a significant risk for development of pelvic inflammatory disease and long-term sequelae such as chronic pelvic pain, ectopic pregnancy, and tubal infertility (2, 3). *C. pneumoniae* primarily causes respiratory infections, with acute pneumonia and bronchitis being the most frequently encountered illnesses (4). However, increasing evidence suggests that this pathogen is strongly associated with chronic diseases such as asthma (5), reactive arthritis (6), and atherosclerosis (7, 8).

The most effective long-term option for control of chlamydial disease is development of a vaccine capable of protecting against infection or severe disease. Extensive knowledge about the major

outer membrane protein (MOMP)³ of *C. trachomatis* as a surface-exposed protein and a target for neutralizing Abs has made it the focus for vaccine development (9–13). Serovar-, subspecies-, and species-specific neutralizing epitopes have been mapped to variable sequence (VS) regions within the protein (11, 12) and may contribute to protective immunity against chlamydial infection. Although synthetic peptides and peptide chimeras representing the VS regions elicit Ab responses, they have been largely unsuccessful in inducing a protective response in animal models (14–16). In a study using polyvalent sera, it was found that absorption with whole elementary bodies (EBs) abrogated neutralizing activity, whereas absorption with peptides representing the VS regions did not (17). It was concluded that the lack of a neutralizing or protective response using synthetic peptides was primarily due to their inability to mimic native epitopes on the chlamydial surface, and thus they could not elicit conformation-dependent immune responses that are essential for serovar-specific clearance (17).

Recently, Zhang et al. (18) have shown that mice immunized with the gene encoding the mouse pneumonitis (MoPn) strain MOMP can elicit both humoral and cellular immune responses to MoPn EB and can confer partial protection against a lung challenge of MoPn. Although these results are encouraging, the approach may be limited in that protection is serovar-specific and does not address the polymorphic nature of the MOMP protein sequence. For an effective vaccine, it is highly desirable that more conserved antigenic determinants on either the MOMP protein or other surface-accessible chlamydial proteins be investigated for broad cross-reactive protection.

The recent identification and characterization of a novel outer membrane protein, PorB, in *C. trachomatis* may provide an attractive alternate to MOMP as a vaccine candidate. Kubo and Stephens (19) have shown that this sequence-conserved 38-kDa protein is localized to the outer membrane surface of chlamydial EBs and

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³ Abbreviations used in this paper: MOMP, major outer membrane protein; VS, variable sequence; EB, elementary body; MoPn, mouse pneumonitis; SPG, sucrose phosphate glutamic acid; KLH, keyhole limpet hemocyanin; IFU, inclusion forming units.

functions as a porin. The authors also found that polyclonal Abs to purified PorB protein had neutralizing properties against chlamydial infectivity in vitro. In this study, we used polyclonal sera raised to peptides representing the *C. trachomatis* PorB sequence and polyvalent immune sera to study the antigenic structure of the PorB protein in detail. The studies were designed to identify antigenic determinants that are on the surface of the intact chlamydial EBs and to characterize which of these are important targets of neutralizing Abs. Furthermore, we tested whether Ags representing the immunoreactive antigenic determinants could inhibit neutralization by PorB antisera and whether peptide-specific neutralizing Abs recognize common Ags in the *C. pneumoniae* PorB amino acid sequence. These antigenic analyses provide an important insight into the potential of PorB Ags as new substrates in chlamydial vaccine testing.

Materials and Methods

Chlamydial strains

C. trachomatis strain D/UW-3/Cx (originally obtained from C.-C. Kuo, University of Washington, Seattle, WA) was grown in HeLa 229 cells and purified by diatrizoate (Renograffin; Squibb Diagnostics, Princeton, NJ) density gradient centrifugation as previously described (20). Briefly, infected cells were disrupted by sonication for 25 s followed by centrifugation at $1,000 \times g$ to remove host cellular debris. Liberated EBs were collected by centrifugation ($12,000 \times g$; 30 min) and resuspended in 10 ml of PBS. The EBs were purified through a 30% diatrizoate column ($58,000 \times g$, 40 min) to separate host cellular components followed by centrifugation (23,000 rpm; 1.5 h) on a discontinuous 30%–44% diatrizoate gradient. After removal of the supernatant, the EB pellet was washed in PBS, resuspended in sucrose phosphate glutamic acid (SPG; 200 mM sucrose, 3.6 mM potassium monophosphate, 8.6 mM sodium phosphate dibasic, 4.9 mM glutamate (pH 7.5)), and stored at -70°C .

Cloning, expression, and purification of PorB

The gene encoding PorB (*porB*) was cloned into the pBAD TOPO-TA vector and transformed into *Escherichia coli* TOP10 competent cells (Invitrogen, Carlsbad, CA) as described by Kubo and Stephens (19). Host cells containing the recombinant PorB plasmid were grown in Luria-Bertani at 37°C until an A_{600} of 0.5 was attained. Expression of PorB as a His-tagged protein was induced by addition of arabinose at a final concentration of 0.02%, and the cultures were incubated for an additional 3 h. PorB was extracted with 1% octylglucoside at 37°C for 1 h followed by dialysis against PBS. The recombinant protein was then purified by nickel column purification under nondenaturing conditions using the His-Bind purification system (Novagen, Madison, WI).

Preparation of synthetic peptide conjugates

Twenty-five overlapping peptides representing the entire PorB sequence were synthesized (Genemed Synthesis, South San Francisco, CA) (Table I). Stock solutions of the PorB peptides (designated B1-1 to B5-5) were prepared in distilled water at a final concentration of 1 mg/ml and stored at

-20°C . The peptides were coupled to Imject maleimide-activated keyhole limpet hemocyanin (KLH; Pierce Endogen, Rockford, IL) at a 1:1 ratio of peptide to KLH according to the manufacturer's instructions. Briefly, 1 mg of maleimide-activated KLH was mixed with 1 mg of PorB peptide in a final volume of 1 ml and incubated for 2 h at room temperature. The conjugated protein was dialyzed against PBS (pH 7.4) for 3 h with three buffer changes. Purified peptide-KLH conjugates were stored at -20°C until used.

Polyclonal immune sera

Human immune sera were obtained from the San Francisco City Clinic from individuals naturally infected with *C. trachomatis*. Polyvalent antisera to *C. trachomatis* serovar B were obtained from rabbits immunized with purified EBs as previously described (21). Monospecific polyclonal antisera to recombinant PorB and synthetic PorB peptide conjugates were produced in Swiss Webster mice (Harlan, San Diego, CA). Five 6- to 8-wk-old female mice were immunized by s.c. injection with 15 μg of purified PorB protein or 100 μg of peptide-KLH conjugate in an equal volume of CFA. Intraperitoneal boost immunizations were performed 2 wk later in IFA. After an additional 2 wk, mice were tested for reactivity to homologous peptide by peptide-specific ELISA. Institutional Review Board approval was obtained for use of human sera and immune sera production in rabbits and mice.

Peptide-specific ELISA

Mouse immune sera to PorB peptides were screened by ELISA using homologous peptide as coating Ag. Polystyrene microtiter plates (Immulon 2; Dynatech, Chantilly, VA) were coated with 50 μl of 5 $\mu\text{g}/\text{ml}$ peptide per well in 50 mM bicarbonate buffer (pH 9.6) and incubated overnight at 37°C . After washing twice with PBS-Tween (PBS; 0.05% Tween 20), the wells were incubated with 100 μl of blocking buffer containing 2% gelatin in PBS for 1 h at 37°C and then washed with PBS-Tween. A 50- μl volume of a 1/1000 dilution of the mouse antisera was added, and the plates were incubated for 1 h at 37°C . The wells were washed three times with PBS-Tween and incubated with a 1/2000 dilution of goat anti-mouse IgG-HRP-conjugated Ab (Zymed Laboratories, South San Francisco, CA) for 1 h at 37°C . After washing three times in PBS-Tween and twice in PBS, the Ab complexes were detected with a mixture of substrate (0.1% hydrogen peroxide) and chromogen (1 mg/ml *o*-phenylenediamine; DAKO, Carpinteria, CA) in 0.1 M citrate buffer. The color was allowed to develop for 15 min and the reaction was terminated by addition of 25 μl of 8N H_2SO_4 . The absorbance at 492 nm was measured on a Titertek Multiscan ELISA plate reader (Flow Laboratories, McLean, VA). Each assay was run in duplicate. Human and rabbit immune sera were tested for reactivity to PorB peptides in a similar ELISA format at 1/1000 dilutions. Samples were treated with either goat anti-human IgG-HRP or goat anti-rabbit IgG-HRP conjugates (Zymed Laboratories) as secondary Ab, and binding to PorB peptide was detected as described above.

Surface accessibility ELISA

The ability of PorB peptide antisera to recognize their cognate Ags on the surface of viable chlamydiae was determined by absorption ELISA. Peptide antisera were adjusted to a dilution corresponding to an A_{492} range of 0.4–1.8 and preincubated for 30 min at room temperature with purified

Table I. Synthetic peptides representing PorB amino acid sequence^a

B1-1-LDAMPAGNPAFPVIPG ^b	B3-1-TSEPLNSESEVTDGMIEVQS
B1-2-FPVIPIGINIEQKNACS	B3-2-GMIEVQSNYGFVWDVSLKKV
B1-3-QKNACSFDLCSYDVL	B3-3-DVSLKKVIWKDGVSVFVGAD
B1-4-NSYDVLALSGLKLC	B3-4-FVGVGADYRHASCPIDYIIA
B1-5-GNLKLCFCGDYIFSEE	B4-1-PIDYIIANSQANPEVFIADS
B1-6-YIFSEEAQVKDVPVVT	B4-2-VFIADSDGKLNFKESVVCVG
B2-1-DVPVVTSVTTAGVGPSPDIT	B4-3-WSVCVGLTIVVNDYVLPYLA
B2-2-PSPDITSTTKTRNFDLVNCL	B4-4-VLPYLAFSIGSVSRQAPDDSF
B2-3-DLVNCLNTNCVAVAFSLPD	B5-1-APDDSEFKKLEDRFTNLKF
B2-4-AFSLPDRSLSAIPLFDVSFE	B5-2-FTNLKFKVRKITSSHRGN
B2-5-FDVSFEVKVGLKQYYRLP	B5-3-SSHRGNICIGATNYVADN
B2-6-QYYRLPMNAYRDFTSEPLNS	B5-4-NYVADNFFYNVEGRWGSQ
	B5-5-GRWGSQRAVNVSGGFQF

^a Residues representing overlapping peptide regions are underlined. All peptides except B1-4 were synthesized with an additional cysteine at their C termini for conjugation to KLH.

^b Residues are listed in single-letter code beginning at the N-terminal end.

chlamydial EBs ($\sim 10^8$ inclusion forming units (IFU)/ml). After centrifugation to remove the EBs, the peptide antisera were tested for residual reactivity to homologous PorB peptide or recombinant PorB as described in the ELISA method above. The difference in reactivity (A_{492}) between the absorbed and unabsorbed peptide antisera was calculated and statistically analyzed by a Student *t* test. A *p* value < 0.05 was considered significant. The absorption experiments were repeated twice.

Chlamydial dot-blot assay

The dot-blot assay was performed as previously described by Zhang et al. (22). Briefly, nitrocellulose membrane (Bio-Rad, Hercules, CA) was pre-soaked in PBS for 10 min and assembled onto a dot-blot apparatus (Bio-Rad). A 50- μ l suspension of chlamydial EBs (5 μ g/ml) in PBS was added to appropriate wells and was allowed to filter by gravity for 10 min followed by vacuum filtration for 5 min to remove all liquid from the wells. The membrane was removed and treated with a blocking solution (2% dried skim milk in PBS) on a rocker for 1 h at room temperature. After three washes in PBS-Tween, the membrane was reassembled. Dilutions of peptide antisera were added to corresponding wells and incubated for 1 h at room temperature. After the wells were washed three times with 200 μ l of PBS-Tween, the membrane was removed from the blotting apparatus and washed three more times with PBS-Tween.

A 1/2000 dilution of goat anti-mouse IgG-HRP conjugate was added to the membrane as secondary Ab and incubated for 1 h at room temperature. After three washes in PBS-Tween, the membrane was treated with ECL detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ) and subjected to autoradiography for 5 s using Kodak X-OMAT AR film (Eastman Kodak, Rochester, NY). Controls included probing immobilized EBs with 1) 2C5, a mAb to the species-specific VS4 region of MOMP, 2) preimmune sera, and 3) anti-Pgp3, an inner membrane chlamydial protein.

In vitro neutralization

In vitro neutralization assays using HaK (Syrian hamster kidney) cells were performed as previously described (23). Serial dilutions of monospecific PorB peptide antisera were prepared in SPG. Mouse antisera produced to

full-length PorB were used as positive controls, and preimmune sera and SPG were used as negative controls. *C. trachomatis* serovar D EBs were diluted in SPG to contain 2×10^4 IFU/ml, and 90 μ l was added to each serum dilution in a final volume of 180 μ l. Neutralization was allowed to proceed for 30 min at 37°C. A total of 50 μ l of each sample was added in triplicate to PBS-washed HaK monolayers and incubated for 2 h at 37°C. After excess inoculum was removed, the cells were rinsed once with PBS, replenished with 200 μ l of RPMI 1640 medium supplemented with 10% FBS and 1 μ g/ml cycloheximide, and incubated at 37°C for 48 h. Chlamydial inclusions were detected by staining with mouse anti-*C. trachomatis* MOMP fluorescent Ab (Wampole Laboratories, Cranbury, NJ) and quantified by counting three fields per well at a magnification of 40 \times . The results were calculated as percentage reduction in mean IFU relative to the control SPG. A 50% or greater reduction in IFU relative to the control was defined as neutralizing. Assays were performed in triplicate.

Results

Ag mapping of PorB with immune sera

The first step in elucidating the structure of PorB was to determine which regions of the protein are antigenic. To define Ags of PorB, the binding of human immune sera, rabbit anti-EB sera, and mouse anti-PorB sera to 25 synthetic overlapping peptides spanning the PorB sequence (Table I) was tested by peptide-specific ELISA. Peptides with high frequencies and high titers of reactivity to the different polyclonal immune sera were considered immunoreactive. Using these criteria, four major antigenic clusters corresponding to Phe³⁴-Leu⁵⁹ (B1-2 and B1-3), Asp¹¹²-Glu¹⁴⁵ (B2-3 and B2-4), Gly¹⁷⁹-Ala²²⁵ (B3-2 to B3-4), and Val²⁶¹-Asn³⁰⁵ (B4-4 to B5-2) were identified (Fig. 1). Each cluster comprised two to three peptides, with higher peaks of reactivity noted for individual peptides B1-3, B2-3, B2-4, B3-4, and B5-2 for all sera tested. The C-terminal end of the protein appeared as a major immunoreactive

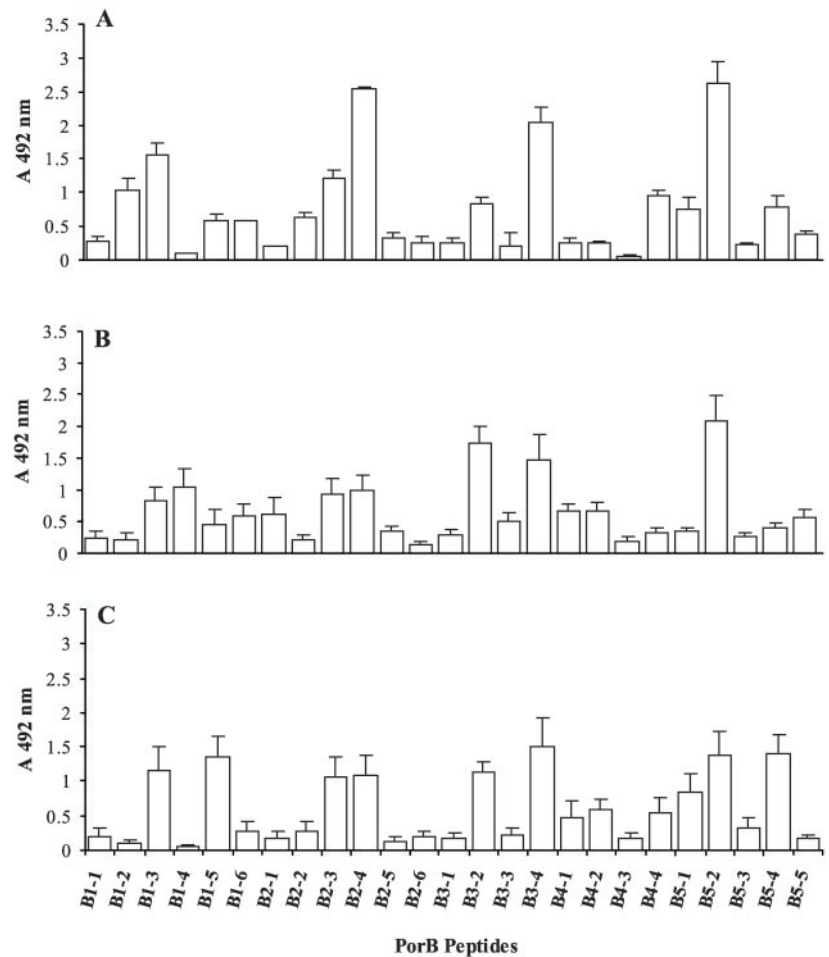


FIGURE 1. Ag mapping of PorB protein. Polyclonal immune sera were used to probe overlapping synthetic peptides representing the entire PorB sequence. Each vertical bar represents the mean absorbance (\pm SEM) of a peptide in the peptide-specific ELISA. Reactivities for pooled rabbit antisera raised against purified chlamydial EBs (A), pooled sera from humans infected with *C. trachomatis* (B), and sera from mice immunized with purified PorB (C) are shown. Sera were tested at a 1/1000 dilution.

region with two antigenic clusters in close proximity. Although variations in individual reactivities exist, all antisera recognized the same determinants, indicating that these antigenic determinants are broadly reactive. Each of the individual sera showed reactivity to purified PorB Ag in a similar ELISA format, confirming that Ab recognition of the PorB peptides was specific (data not shown). Except for peptide B2-3, which contained a stretch of hydrophobic residues in its C-terminal region, most of the reactive Ags were hydrophilic. The hydrophilicity and reactivity of the immunoreactive peptides suggest that they may be surface accessible and targets of neutralizing immune responses.

Determination of surface accessibility of PorB Ags

Neutralization of chlamydial infectivity requires that antigenic determinants are surface exposed and accessible for Ab recognition (17, 24). The experimental approach to determining whether the identified PorB Ags are surface accessible was to generate a panel of mouse antisera to each of 25 overlapping synthetic peptides representing PorB (Table I) and to use these to probe for their cognate Ag on chlamydial EBs in absorption ELISA and EB surface-specific dot-blot assays.

Characterization of anti-peptide conjugate Abs. A panel of mouse antisera raised to overlapping synthetic PorB peptides was evaluated for reactivity by a peptide-specific ELISA. All sera reacted with their homologous peptides with mean end-point titers (\log_2 values) ranging from 9 to 17 (Table II). The reactivity of peptide-specific antisera to recombinant PorB or homologous peptides was reduced 58–95% by competitive inhibition with respective peptides, demonstrating that peptide recognition was specific

(data not shown). In contrast, the peptide sera were unaffected by absorption with KLH and an unrelated chlamydial peptide CT673 (data not shown). Because MOMP quantitatively predominates the surface of chlamydial EBs and is known to mediate neutralization of infectivity, the panel of peptide antisera was tested for cross-reactivity to this protein. No cross-reactivity of the peptide-specific antisera was observed in that reactivity to recombinant MOMP protein was $<0.3 A_{492}$ for all sera tested (Table II).

EB absorption studies. To determine which of the peptide-specific Abs were directed at PorB Ags on the chlamydial EB surface, absorption studies were conducted in an ELISA format. Peptide-specific antisera were preincubated with viable EBs before testing for reactivity to PorB peptides and recombinant PorB. If absorption with intact EBs reduced the reactivity of peptide-specific Ab compared with the unabsorbed sera, it can be inferred that the absorbed Abs were directed toward surface-accessible antigenic determinants on PorB. Absorption of sera raised to peptides B1-2, B1-3, B2-1, B2-3, B2-4, B3-2, B3-4, and B5-2 with chlamydial EBs resulted in a significant decrease in reactivity to their cognate peptides (Fig. 2; $p < 0.05$). Antisera B2-3, B3-2, B3-4, and B5-2 showed the largest decreases in reactivity when absorbed with EBs. In contrast, antisera B1-6 and B2-6, which showed high reactivity with their respective peptides (Table II), showed no decreases in reactivity when absorbed with EBs. This verified that the chlamydial EBs were intact during absorption. Reactivity of peptide-absorbed antisera to recombinant PorB revealed similar profiles of surface-exposed antigenic determinants, indicating that the peptide-specific Abs bind to cognate peptides as well as whole protein (data not shown). When polyclonal immune sera from humans, rabbits, and mice were preabsorbed with EBs and tested for reactivity with PorB peptides, a decrease in reactivity was observed for the same peptides (Fig. 3). Although the baseline reactivity to peptides B1-2 and B2-1 in mice is low (Fig. 1), the absorption results show that these peptides are surface accessible. The data from EB absorption studies supported the specificity of the peptide-Ab interaction and provided evidence that the immunoreactive PorB antigenic determinants are surface exposed.

Dot-blot analyses. To confirm the specificity and surface reactivity of the PorB peptide antisera, a dot-blot analysis was performed with chlamydial EBs as previously described by Zhang et al. (22). Anti-B2-1, B2-3, B3-4, and B5-2 showed the strongest reactivities to chlamydial EBs, followed by anti-B1-2, B1-3, B2-4, and B4-2 (Fig. 4). This demonstrates that the cognate antigenic determinants for these Abs are exposed on the bacterial surface. Peptide B3-2, which showed surface accessibility by the EB absorption method, did not show a strong signal in the dot-blot assay. In contrast, peptide B4-2, which was not surface exposed by EB absorption, gave a strong signal by the dot-blot method. The absence of a reactive signal for the remaining PorB peptide antisera implies that their respective antigenic determinants are inaccessible for Ab recognition. When the EBs were treated with SDS, the dot-blot profile for all the peptide antisera was strong, indicating that SDS had solubilized the outer membrane of the EB, and all PorB Ags were consequently accessible (Fig. 4). Preimmune sera were negative for reactivity with both SDS-treated and untreated EBs. An additional control was included in which sera to a nonsurface protein, Pgp3 (25), were used to probe immobilized EBs. This Ab did not bind to the intact EBs but showed strong reactivity with lysed EB, verifying the structural integrity of the viable EB. The findings in the dot-blot analyses were consistent with those of the EB absorption studies and confirmed that the major antigenic determinants of PorB are surface exposed.

Table II. Immunological properties of mouse antisera raised against PorB peptide conjugates

Ab	Homologous Peptide ELISA Reciprocal Titer (\log_2) ^a	Cross-reactivity with rMOMP ELISA (A_{492}) ^b
B1-1	15 ^c	0.24
B1-2	13	0.18
B1-3	13	0.09
B1-4	15	0.03
B1-5	16	0.19
B1-6	16	0.00
B2-1	13	0.00
B2-2	15	0.30
B2-3	12	0.00
B2-4	14	0.00
B2-5	15	0.04
B2-6	17	0.27
B3-1	12	0.21
B3-2	12	0.16
B3-3	11	0.07
B3-4	17	0.22
B4-1	11	0.14
B4-2	11	0.01
B4-3	13	0.27
B4-4	14	0.19
B5-1	11	0.11
B5-2	13	0.02
B5-3	11	0.19
B5-4	9	0.02
B5-5	11	0.00
Anti-rMOMP ^d	n/a	2.31

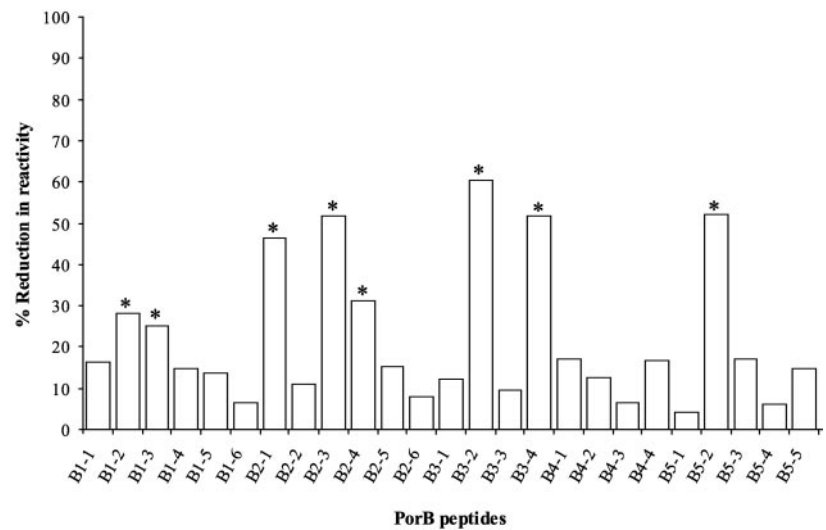
^a Serial 2-fold dilutions of peptide antisera were made and tested for reactivity to their homologous peptides at 5 $\mu\text{g}/\text{ml}$. Absorbance values were subtracted from background (range 0.05–0.160).

^b Peptide antisera were diluted 1/1000 and tested for reactivity to rMOMP. Absorbance values for background (range 0.05–0.160) were subtracted from the peptide values.

^c Groups of five mice were immunized for each peptide.

^d Anti-rMOMP sera were obtained from mice immunized with rMOMP and tested at 1/1000 for reactivity to relevant Ag at 5 $\mu\text{g}/\text{ml}$.

FIGURE 2. Accessibility of PorB antigenic determinants on the surface of chlamydial EBs. Pretitrated mouse peptide antisera were preincubated with purified chlamydial EBs and tested for residual reactivity to homologous peptide. The results are presented as percent reduction in serum reactivity (A_{492}) to PorB peptides due to absorption with viable EBs. *, Significance at $p < 0.05$ when absorbed sera were compared with unabsorbed sera by a Student's t test.



In vitro neutralization of chlamydial infectivity

In vitro neutralization assays are a central component in evaluating functionality of chlamydial immune responses and provide a correlate of protective immune responses (23). The neutralization data for the peptide antisera revealed four regions of neutralizing activity on the PorB protein, with 50% reciprocal neutralization end-point titers ranging from $\sim 1:32$ to $\sim 1:2048$ (Fig. 5). Anti-B1-3, B2-4, and B5-2 sera provided the strongest neutralization activities, with 50% reciprocal end-point titers $> 1:1024$, followed by anti-B2-3, B3-2, B3-4, and B4-2. Notably, these regions of neutralizing activity overlap regions identified as immunoreactive and surface exposed by ELISA and dot-blot analysis. This confirms that the antigenic determinants contributing to the neutralizing property of PorB antisera are surface exposed. When a pool of

peptides representing the strongly neutralizing antigenic determinants (B1-3, B2-3, B2-4, B3-2, B3-4, B4-2, and B5-2) was used in a competitive inhibition assay with PorB antisera, the neutralizing ability of PorB antisera was markedly reduced (Table III). Inhibition of neutralization was concentration dependent, with $10 \mu\text{g}$ of peptides completely blocking neutralization. Similarly, preincubation of the PorB antisera with purified recombinant PorB also ablated neutralization of chlamydial infectivity. Heat denaturation of PorB appeared to have no additional effect on inhibiting PorB antisera because the neutralization results were similar to that observed when intact PorB was used as the inhibiting Ag. These results revealed that neutralizing PorB antisera contain a population of Abs that recognize predominantly linear antigenic determinants on the surface of the EB.

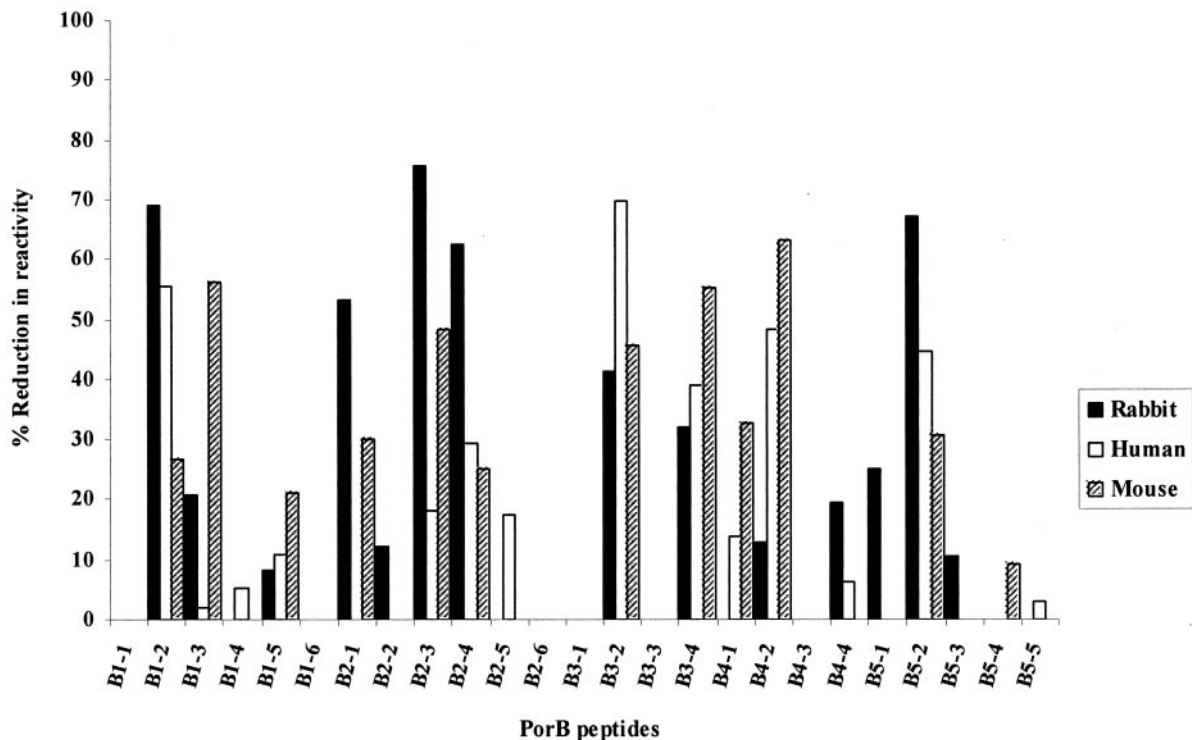
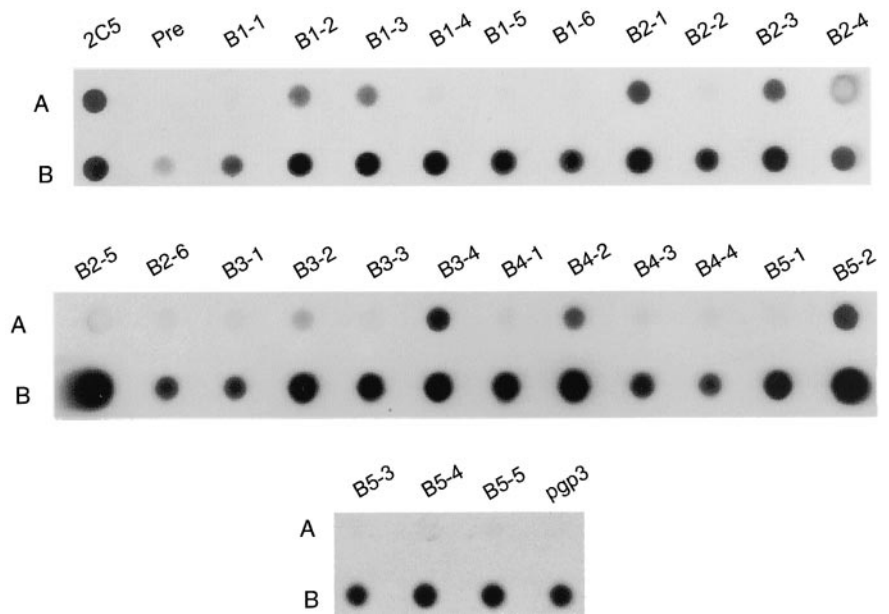


FIGURE 3. Change in reactivities of EB-absorbed polyclonal immune sera to PorB peptides. Rabbit, human, and mouse immune sera were preincubated with chlamydial EBs and assayed for residual reactivity to PorB peptides by ELISA. The results are presented as percent reduction in serum reactivity (A_{492}) due to absorption with viable EBs. Sera were tested at a $1/1000$ dilution.

FIGURE 4. Specificity and surface reactivity of PorB peptide antisera as determined by dot-blot analysis. Peptide-specific antisera were used to probe viable (A) or SDS-treated (B) chlamydial EBs immobilized on a nitrocellulose membrane. Peptide antisera and preimmune negative control sera were tested at a 1/100 dilution. The Pgp3-specific control antisera were used at a 1/500 dilution, and the positive control mAb to MOMP, 2C5, was tested at a 1/2000 dilution.



Identification of common PorB antigenic determinants between C. trachomatis and C. pneumoniae

Comparison of the PorB amino acid sequences for *C. trachomatis* and *C. pneumoniae* revealed 59.3% identity, indicating that this protein is conserved between species (19). To determine whether the neutralizing *C. trachomatis* PorB peptide antisera recognized common Ags between the two species, the reactivity of the neutralizing PorB peptide antisera to analogous synthetic *C. pneumoniae* PorB peptides was evaluated. Four *C. pneumoniae* PorB peptides (CPn 1-3, CPn 3-2, CPn 4-4, and CPn 5-2) showed strong reactivities to the corresponding *C. trachomatis* peptide antisera (Table IV). Amino acid sequence comparison revealed that *C. pneumoniae* PorB peptides with the highest cross-reactivity (CPn 3-2, CPn 4-4, and CPn 5-2) shared a minimum of 67% residue identity (bold) and 75% sequence similarity with their corresponding *C. trachomatis* PorB peptides. In contrast, peptides with <50% sequence identity (CPn 1-2, CPn 2-3, CPn 2-4) showed little or no cross-reactivity with the corresponding peptide antisera. Ab recognition of *C. pneumoniae* PorB antigenic determinants was not

always associated with an increased number of shared or similar residues. For instance, CPn 4-3, which has 60% identity and 90% similarity with B4-3, showed no reactivity with the corresponding B4-3 antisera, whereas CPn 2-4 with 40% identity and only 50% similarity showed moderate cross-reactivity. The reactivity patterns suggest that these *C. pneumoniae* antigenic determinants may be surface exposed and may also be the targets of neutralizing Ab responses. Collectively, these results show that neutralizing PorB peptide antisera recognize similar PorB Ags in *C. trachomatis* and *C. pneumoniae* and suggest that the sera may have broad neutralizing properties directed against surface-exposed peptides.

Discussion

Protection from chlamydial infections by the host immune response requires specific recognition of unique antigenic determinants that are exposed on the surface of native EBs and are capable of eliciting neutralizing Abs (12, 17, 26). To date, MOMP is the only known surface-exposed Ag that mediates neutralization of chlamydial infectivity and confers protection (17, 22). However,

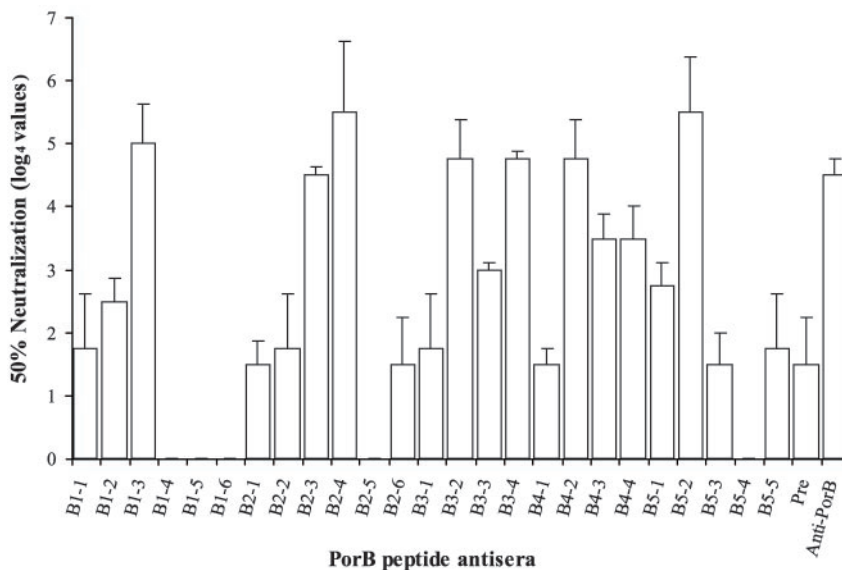


FIGURE 5. Sera titer representing 50% neutralization of infectivity (\pm SEM) for *C. trachomatis* EBs by PorB peptide antisera. EBs were incubated with serial dilutions of peptide antisera and added to HaK cells. IFU were counted and the percent neutralization for each dilution relative to the diluent SPG control was determined.

Table III. Percentage of neutralization of chlamydial infectivity by PorB antisera in the presence of inhibitory Ag

Ag	Concentration (μg) ^a		
	2 (%)	5 (%)	10 (%)
Peptides ^b	28 ^c	0	0
PorB	39	11	0
Denatured PorB ^d	39	6	11

^a Increasing concentrations of inhibiting Ag were preincubated with a dilution of PorB antisera yielding ~60% neutralization used in in vitro neutralization assays as described in *Materials and Methods*.

^b A pool of synthetic peptides representing Ags that are neutralizing targets were used for inhibition studies.

^c The percentage of neutralization was calculated for each sample relative to the SPG control. Assay was done in triplicate.

^d Purified PorB protein was denatured by boiling for 10 min.

development of a MOMP-based vaccine has been challenging because the neutralizing determinants are antigenically variant (12) and protection is conformation dependent (17). Consequently, there has been a high priority to identify other chlamydial proteins as alternate candidates.

The genome sequence of *C. trachomatis* revealed several new outer membrane proteins including a family of sequence variant polymorphic membrane proteins of unknown function and PorB, an outer membrane porin (27). Recently, it has been shown that PorB antisera are also efficient at neutralizing chlamydial infectivity (19). Although this finding is encouraging, the topology and neutralizing Ags of the protein are unknown. Synthetic peptides have been extensively used to map antigenic determinants of chlamydial MOMP (12, 28, 29) and have provided additional information that correlates amino acid sequence with variation in the MOMP VS regions (30). In the present study, a panel of synthetic peptides, polyclonal immune sera, and peptide-specific antisera were used to precisely map the antigenic structure, surface accessibility, and neutralizing properties of PorB. A schematic model of the predicted secondary structure of PorB depicts a protein consisting of 16 transmembrane anti-parallel β -sheets with the immunoreactive antigenic determinants protruding into the extracellular matrix (Fig. 6). This is consistent with the structure of the *E. coli* porin OmpA (31) and the predicted structure of the chlamydial porin MOMP (12, 32). Four regions of the protein were identified as strongly antigenic, and the antigenic determinants that comprise

Table IV. Reactivity of neutralizing *C. trachomatis* PorB peptide-specific antisera to synthetic *C. pneumoniae* PorB peptides

Peptide	Amino Acid Sequence ^a	Reactivity to Peptide Antisera ELISA (A_{492}) ^b
CPn 1-2	APVLPGVNPEQ ET GWCA	0.10
CPn 1-3	QTGWCA FQ LCNSYDLF	1.24
CPn 2-3	DLN SS ISSSCVFATIALQE	0.49
CPn 2-4	TTALQ ET SPA AI PLLDIAFT	0.58
CPn 3-2	GLIEVQSDYGI VW GLSLQKV	2.76
CPn 3-3	GLSLQKV LW KDGV SF VGV SAD	0.19
CPn 3-4	FVGV SAD YRHG SS PI NY IIV	0.50
CPn 4-2	IYFDAT DGN LSY KE WSASIG	0.82
CPn 4-3	WSASIG IST YL ND YVLPYAS	0.12
CPn 4-4	VL PY AS VS IGN TSR K AP SD SF	3.00
CPn 5-2	F T N F K F K I R K I T N F D R V N	2.08

^a Synthetic peptides analogous to *C. trachomatis* PorB peptides were synthesized as described in *Materials and Methods*. Residues are listed in single-letter code beginning at the N-terminal end. Regions of shared amino acid sequences are in bold.

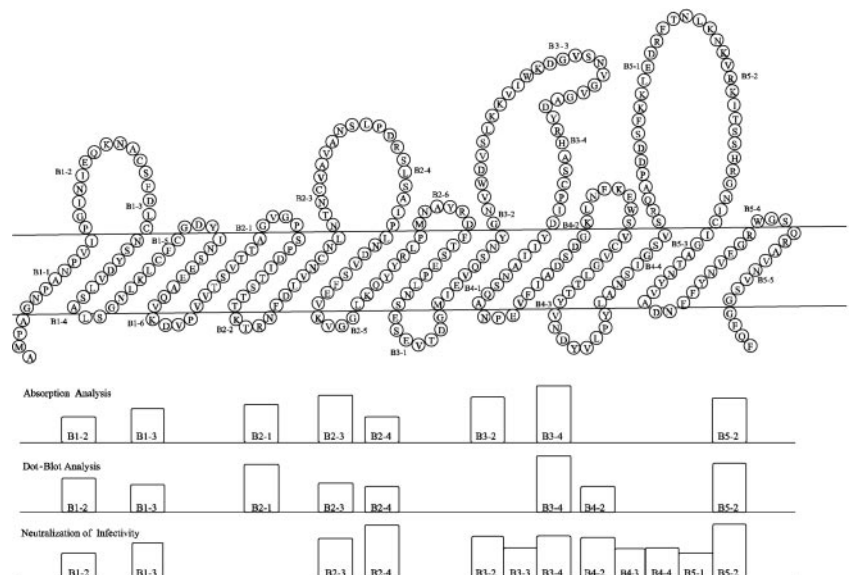
^b Peptide-specific antisera were diluted 1/1000 and tested for reactivity to *C. pneumoniae* PorB peptides at 5 $\mu\text{g}/\text{ml}$. Absorbance values at 492 nm were compared to values for reactivity of antisera to cognate *C. trachomatis* peptides.

these clusters were predominantly hydrophilic and predicted to be surface exposed.

Serum absorption experiments (28, 29) and dot-blot analyses (22) with viable chlamydial EBs have been particularly useful in determining the distribution of MOMP epitopes on the chlamydial EB surface. These methods were similarly used to identify PorB antigenic determinants that are surface exposed on chlamydial EBs, using PorB peptide antisera and polyclonal immune sera. With the exception of two antigenic determinants, the absorption and dot-blot studies with peptide antisera consistently identified the same seven antigenic determinants as surface exposed (Fig. 6). The discrepancy observed for B3-2 and B4-2 may be attributed to fundamental differences between the ELISA and the dot-blot methods or differences in the peptide-specific Ab populations being recognized. Nevertheless, when polyclonal immune sera from humans and rabbits were used in similar EB absorption studies, both antigenic determinants were recognized as surface accessible.

Whereas the absorption and dot-blot analyses delineated surface-accessible PorB antigenic determinants, use of the standardized in vitro neutralization assay established by Byrne et al. (23)

FIGURE 6. Model of secondary structure for *C. trachomatis* PorB protein. PorB secondary structure was deduced by analogy to substrate-specific porins and Chou-Fasman prediction for turn-promoting residues (31). Peptides exposed on the surface of chlamydial EBs as determined by absorption ELISA and dot-blot analysis and peptides that elicit neutralizing Ab responses are indicated by bars below.



has provided valuable insight into the potential of PorB Ags in a protective anti-chlamydial response. The neutralization profile developed with PorB peptide antisera reveals four major regions of neutralizing activity that overlap the regions identified as immunoreactive and surface exposed (Fig. 6). The proximity of the neutralizing Ags supports the existence of a complex of discontinuous antigenic structures that contribute to the PorB-specific neutralizing immune response. Because Abs raised to synthetic peptides may be limited in their recognition of linear vs conformational antigenic determinants on the native Ag, caution must be exercised in interpreting the PorB neutralization data. Although the complex of discontinuous antigenic structures as defined by the peptide-specific antisera does not exclude the presence of conformational antigenic determinants, evidence provided in this study that the neutralizing activity of PorB antisera is ablated by synthetic PorB peptides or purified PorB suggests the presence of a large population of PorB Abs whose neutralizing activity is independent of conformational requirements and will facilitate evaluation of PorB polypeptides as potential immunogens.

Identification of the major neutralizing antigenic determinants for PorB has important implications for chlamydial vaccine design. In addition to having surface-accessible and possibly conformation-independent antigenic determinants, a major advantage of PorB is that it is highly sequence conserved between serovars and species (19) and can be expected to provide protection for a broad spectrum of chlamydial strains including *C. pneumoniae*. Consistent with this is the finding that neutralizing Abs to *C. trachomatis* serovar D PorB Ags cross-react with analogous *C. pneumoniae* peptides, and they also neutralize infectivity of *C. trachomatis* serovar B (data not shown). Moreover, the *C. pneumoniae* PorB peptides used in this study have hydrophilicity profiles similar to those of their *C. trachomatis* counterparts, increasing the likelihood that these Ags are also surface oriented on the native Ag and are targets of a broadly neutralizing Ab response. PorB as a vaccine Ag will obviate the need to incorporate serovar- and species-specific determinants and, if effective, will be valuable in providing protection against multiple serovars or species, which is highly desirable for long-term control of chlamydial infections.

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