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MAPPING OF MONOCLONAL ANTIBODY BINDING SITES ON CNBr FRAGMENTS OF THE S-LAYER PROTEIN ANTIGENS OF *RICKETTSIA TYPHI* AND *RICKETTSIA PROWAZEKII**

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Abstract—The 120 kDa surface protein antigens (SPAs) of typhus rickettsiae lie external to the outer membrane in regular arrays and chemically resemble the S-layer proteins of other bacteria. These proteins elicit protective immune responses against the rickettsiae. In order to study the immunology of these proteins, purified SPAs from *Rickettsia typhi* and *Rickettsia prowazekii* were fragmented with CNBr. The fragments were separated by SDS-PAGE and were recovered on PVDF membrane following electroblotting. The origin of eight major fragments from *R. prowazekii* and seven major fragments from *R. typhi* was determined by automated N-terminal amino acid sequencing and by comparison with the DNA sequence encoding *R. prowazekii* SPA. The cleavage patterns and protein sequences of the two proteins differed significantly. CNBr fragments corresponding to the C-terminus (amino acid 1372-1612 of the deduced sequence from encoding gene *spaP*) were not present in both SPAs. This suggests that the corresponding C-terminal region was not synthesized or was removed during SPA translocation to the cell surface. Modified amino acids were detected in each protein. Eighteen monoclonal antibodies selected for varied reactivity with both native and denatured SPA proteins could be classified into eight different types based on western blot analysis of the CNBr fragments. Six of the monoclonal antibody types reacted predominantly with a single region of the SPAs. Two types of antibodies bound to several CNBr fragments which contained both limited sequence similarity and modified amino acids either of which might account for the multisite binding of these antibodies.

INTRODUCTION

Rickettsia prowazekii and *Rickettsia typhi*, respectively, the etiologic agents of epidemic and endemic typhus, are obligate intracellular bacteria. They possess a crystalline surface layer (S-layer) as the outermost component of their cell envelope (Palmer *et al.*, 1974; Ching *et al.*, 1990). S-layers have been found on the surface of many gram positive and negative bacteria and archaeobacteria and are composed of protein or glycoprotein subunits which are arranged on the cell surface in a regularly repeating hexagonal, tetragonal, or linear pattern (Reviewed extensively by Baumeister *et al.*, 1988; Hovmiller *et al.*, 1988; Sleytr and Messner, 1983, 1988). Such assembled arrays may serve as protective barriers against proteolytic enzymes or bacteriophage, or act as ion traps

for charged metabolites. Some S-layer proteins are implicated in cell adhesion and surface recognition, others determine cell shape and envelope rigidity, and some even appear to mediate exchange of genetic material. S-layer proteins are also important virulence factors for several pathogenic gram-negative bacteria (Kay *et al.*, 1984; Pei *et al.*, 1988).

The S-layers of *R. prowazekii* and *R. typhi* have been isolated and characterized as the 120 kDa species-specific surface protein antigens (SPAs) (Dasch, 1981; Dasch *et al.*, 1981; Ching *et al.*, 1990). Two homologous large protective antigens have been identified in *Rickettsia rickettsii* (Anderson *et al.*, 1990; Gilmore *et al.*, 1989) and *R. conorii* (Vishwanath *et al.*, 1990). Antigenically related proteins are also present in *R. canada* (Dasch and Bourgeois, 1981) and all of the other spotted fever group rickettsiae examined so far except *Rickettsia bellii* (Dasch *et al.*, 1990). *R. typhi* SPA and *R. prowazekii* SPA each constitutes 10-15% of the total cellular protein and is readily released by shaking the rickettsiae in hypotonic solution. This treatment results in the loss of the repetitive subunits from the rickettsial outer membrane giving it a smooth outer appearance (Ching *et al.*, 1990). Although this ease of extraction is relatively unusual for an S-layer, as strong chaotropic ions or detergents are often used for their release (Koval and Murray, 1984), the large amount and high degree of purity of this high molecular weight rickettsial protein are exactly as expected for an S-layer forming a

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The experiments reported herein were conducted according to the principles set forth in the *Guide for the Care and Use of Laboratory Animals*, Institute of Laboratory Animal Resources, National Research Council, DHHS Publication (NIH) 86-23 (1985).

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monomolecular layer around the entire microorganism (Baumeister *et al.*, 1988). As described for other S-layer proteins, the SPAs of these typhus rickettsiae contain a high proportion of acidic, hydroxy and hydrophobic amino acids but are somewhat unusual in containing cysteines (Ching *et al.*, 1991; Dasch *et al.*, 1985). Both theoretical analysis of the amino acid sequence deduced from the DNA sequence and direct physical chemical measurements on purified SPAs of the typhus rickettsiae indicate that these S-layer proteins are very rich in beta sheet structure but have little alpha helical structure (Ching *et al.*, 1991).

The SPAs of the typhus rickettsiae are highly immunogenic in humans and animals and have been shown to be responsible for the species-specific serological reactions of the typhus group rickettsial antigens (Bourgeois and Dasch, 1981; Dasch and Bourgeois, 1981; Dasch *et al.*, 1981; Misiti and Dasch, 1985). A variety of cell-mediated immune responses to the rickettsiae can be stimulated with SPA (Carl and Dasch, 1989). The typhus SPAs also elicit strong protective responses against the rickettsiae in guinea pigs and mice (Bourgeois and Dasch, 1981; unpublished observations). The gene encoding the SPA of *R. prowazekii* (*spaP*) has recently been cloned, sequenced and expressed in *E. coli* (Carl *et al.*, 1990; Dobson *et al.*, 1991). The encoded protein has a molecular weight of 169 kDa, far larger than any of the physical estimates obtained directly from isolated SPA (Ching *et al.*, 1991). Because of the obvious discrepancies in SPA molecular weight, and the possibility that rickettsial SPAs may be modified by host cell or rickettsial enzymes as occurs with the 17 kDa rickettsial lipoprotein (Anderson *et al.*, 1988), we decided to study directly the immunochemistry of the SPAs extracted from the rickettsiae. Due to the large size of the SPAs, CNBr fragmentation at methionine residues was used to obtain a limited number of fragments that could be more easily manipulated. Fragments of both SPAs were resolved on SDS-PAGE, identified by amino acid sequencing and then analyzed by Western blotting using 18 SPA monoclonal antibodies. A number of specific and cross-reactive epitopes were localized on different fragments of SPAs. In addition, evidence was obtained for post-translational modification of the SPAs and for the incomplete expression (or truncation by proteases) of 200–300 C-terminal amino acids encoded by the *spaP* gene.

MATERIALS AND METHODS

Preparation of CNBr fragments from SPA

SPA was obtained from Renografin density gradient purified *R. typhi* strain Wilmington or *R. prowazekii* strain Breinl by two or three successive extractions with distilled water at 4°C (Dasch, 1981). The pooled extracts were ultracentrifuged at 200,000 *g* for 2 hr, the supernatant was sterilized by passage through a 0.45 µm membrane filter, and the filtrate was stored in aliquots at 4°C or at –60°C. Cleavage of the protein at methionine residues with CNBr was carried out according to

the method of Gross (1967). One volume of protein solution was diluted with three volumes of 98% formic acid. Freshly made 1 M CNBr in 75% formic acid (1000-fold molar excess over the estimated methionine content) was added (about 1 mg/ml final protein concn). The mixture was incubated at room temp for 24 hr. Ten volumes of H₂O were then added to the reaction mixture, the sample was aliquoted into several tubes, and the tubes were dried completely with a rotary speed-vac apparatus (Savant Instruments). Dried samples were stored desiccated at –20°C. Prior to analysis of SDS-PAGE, the samples were dissolved in 8 M urea, diluted with an equal volume of H₂O, then mixed with sample solubilizer (Laemmli, 1970) at final concns of 1 µg/ul of protein, 62.5 mM Tris-HCl, pH 6.8, 5% β-mercaptoethanol, 2% SDS, 10% glycerol, 2 M urea, 0.01% bromophenol blue, and heated at 60°C for 10 min.

Gel electrophoresis and electroblotting

CNBr derived protein fragments were separated by SDS-PAGE either on large gels (16 cm × 13 cm × 0.75 mm) or on mini gels (8.2 cm × 7.2 cm × 0.75 mm). For the large gels, a linear gradient in bis-acrylamide cross-linker was made from 9 ml of gel solution A and 9 ml of gel solution B. Gel solution A contained 16% acrylamide, 0.53% bis-acrylamide (acrylamide:bis-acrylamide 30:1), 1 M Tris-HCl, pH 8.45, 0.1% SDS. Gel solution B had the same composition except for the bis-acrylamide (1.06% vs 0.53%). A 4% acrylamide stacking gel (same cross-linkage as gel solution A but in 0.75 M Tris-HCl, pH 8.45, 0.1% SDS) was formed on top of the gradient gel. Electrophoresis was carried out in a Bio-Rad Protein II apparatus using 0.1 M Tris, 0.1 M Tricine, 0.1% SDS, pH 8.25 as the cathode buffer and 0.2 M Tris-HCl, pH 8.9 as the anode buffer (Schagger and von Jagow, 1987). The gel was pre-run for 2 hr at 20 mA. Electrophoresis was continued for 16 hr at 20 mA per gel in a cold room. For mini gels, a step gradient in acrylamide concn was made of 2.4 ml of gel solution A, 0.8 ml of 10% acrylamide solution with the same cross-linker and buffer as gel solution A, and 1.2 ml of 4% stacking gel solution. The gel was pre-run for 50 min. Samples were electrophoresed for 140 min at 30 mA gel in a Bio-Rad Mini-Protein II Dual Slab Cell. Samples were loaded at 10 µg well, 300–350 µg per large gel, or 100–200 µg per minigel. Protein fragments separated on the gel were electroblotted onto PVDF paper (Millipore, Milford, CT) for 3 hr at 0.3 A (Hoefer model TE50) in 25 mM sodium phosphate, pH 7.5 (transfer buffer). Protein fragments on the electroblotted PVDF paper were either stained directly with Coomassie blue or detected by blot immunoassay.

Monoclonal antibodies

Mouse hybridoma cell lines were prepared by conventional HAT selection techniques following polyethylene glycol mediated cell fusion. SP2 0-Ag14 plasmacytoma cells were fused with spleen cells from BALB/cJ or NMRI mice which had been hyperimmunized with either Renografin density gradient purified *R. typhi*

strain Wilmington, *R. prowazekii* strains Breinl or Madrid E. or the purified 120 kDa SPAs of those species (Raoult and Dasch, 1989). The hybridoma cells were screened by ELISA and cloned by limiting dilution. The antibodies secreted by the selected monoclonal hybridoma cells were then characterized by Western blotting and biological assays. Monoclonal antibodies were used as undiluted tissue culture supernatants of the cells grown in RPMI 1640 with 10% fetal bovine serum. The supernatants were stored at 4 C with 0.02% Thimersol as preservative. None of these antibodies exhibited protection in a mouse passive transfer experiment (toxin neutralization) (Dobson *et al.*, 1989).

Blot immunoassay

Immobilon membrane with electroblotted CNBr fragments of SPA were rinsed with HPLC grade water and stored for later use or cut directly into strips. Alternate strips were either stained with Coomassie blue to detect the presence of protein fragments or subjected to immunoassay for the detection of monoclonal antibody binding epitopes. Strips were blocked with 5% non-fat dry milk in TBS (10 mM Tris-HCl, 250 mM NaCl, pH 7.5) for 1 hr to prevent nonspecific antibody binding. In the immunoassay the strips were incubated with 2 ml of antibody (monoclonal culture supernatants undiluted, in an incubation tray with 25 milled 150 x 10 x 10 mm wells) and incubated for 2 hr at 30°C. After removal of the antibody, strips were washed three times in TBS for 10 min each. Anti-immunoglobulin-horseradish peroxidase conjugates (Bio-Rad Laboratories, Richmond, CA)

were diluted 1:1000-1:3000 in 3% milk in TBS and incubated with the strips for 1 hr at 30°C. After three times of 10 min washes in TBS the strips were incubated in 0.015% 4-chloro-1-naphthol and 0.015% hydrogen peroxide in 16.7% methanol in TBS for 10-15 min (Hawkes *et al.*, 1982). Finally, the strips were thoroughly rinsed in water, dried between filter paper and photographed.

N-terminal amino acid sequence analysis

Following purification by SDS-PAGE and electroblotting onto PVDF membranes, CNBr fragments were subjected to automated sequence analysis on a Model 477A sequencer equipped with an on-line PTH analyzer Model 120A (Applied Biosystems, Foster City, CA) (Matsudaira, 1987).

RESULTS

Generation of CNBr fragments of SPA

About 20-40% of the SPAs obtained by hypotonic shock exist as disulfide linked polymers of 120 kDa monomers (Dasch *et al.*, 1985), while a different form of each SPA which migrates more rapidly than monomer on native gels was obtained from French pressure cell extracts of the rickettsiae (Dasch *et al.*, 1981). The faster electrophoretic forms lacked the disulfide linked polymers and migrated at about 117 kDa by SDS-PAGE, suggesting they originated from limited proteolytic digestion of the original SPAs. Similar "nicked" forms of both SPAs can be obtained directly by treatment of the

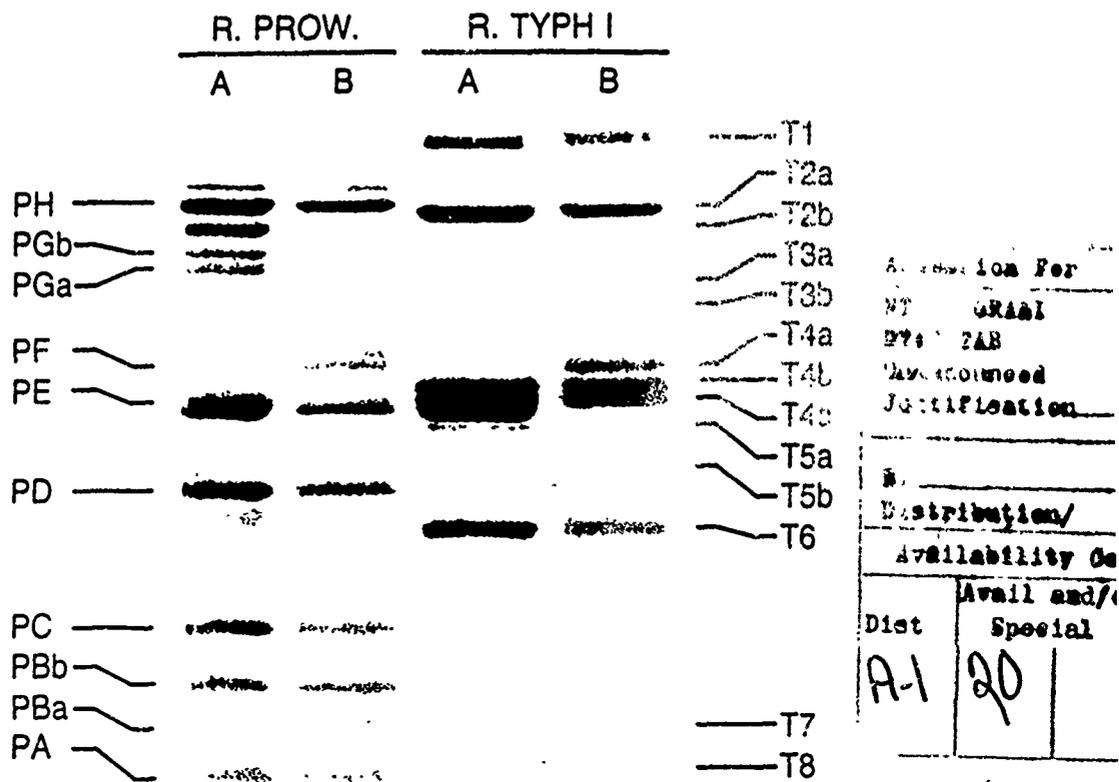


Fig. 1. Coomassie blue stained mini gel electrophoresis pattern of CNBr-derived fragments of SPA from *R. prowazekii* and *R. typhi*: 10 µg of total protein fragments was loaded for each lane. Lane A: protease nicked SPA preparations. Lane B: Intact SPA preparations.



hypotonic shock-released SPAs with commercial preparations of trypsin or chymotrypsin (Ching and Dasch, in preparation). We compared the Coomassie stained CNBr fragment patterns of nicked and intact SPAs from both *R. typhi* and *R. prowazekii* (Fig. 1). For all four SPA preparations the size of the fragments ranged from a few kDa to more than 60 kDa. Some fragments have very similar sizes, e.g. PGb and PGa, PBb and PBa, and especially T4a, T4b, and T4c. We tried many different gel systems in order to separate these closely migrating fragments. The 16% acrylamide with 30:1 vs 15:1 bisacrylamide gradient gel, as described under materials and methods gave us the best resolution. Ten and 13 major fragments (labeled in Fig. 1) were reproducibly obtained from different preparations of the nicked and intact SPAs of *R. prowazekii* and *R. typhi*, respectively. The *R. typhi* and *R. prowazekii* CNBr fragment patterns

were obviously different from each other, but several fragments derived from the intact SPAs had similar mol. wts (PBa and T7; PF and T4a). Supporting the idea that these fragments were derived from homologous regions of the SPA molecules, these four fragments (as well as T3a and T2b) were each absent in the nicked SPAs, while all the other major fragments were present in both nicked and intact SPA preparations. Because the CNBr fragmentation patterns of *R. typhi* and *R. prowazekii* SPAs differ substantially, the number and location of many methionines are not highly conserved between the two SPAs.

Identification of CNBr fragments of SPA

The *N*-terminal sequences and apparent mol. wts of many of the CNBr fragments, purified by electroblotting onto PVDF membrane and excision, were determined

Table 1. Characteristics of CNBr-derived fragments of *Rickettsia prowazekii* and *Rickettsia typhi* SPAs

Fragment	Estimated from SDS-PAGE (kDa)	Expected from ^a gene sequence (aa residues) (kDa)	Comparison of sequence of <i>R. prowazekii</i> SPA deduced from its DNA sequence with the partial <i>N</i> -terminal amino acid sequences of SPA CNBr fragments ^b
PE	22.5	21.5 (6-211) QYNRTTNA AATTFDGI GFD
T4b	23.5	21.5 (6-211)?.....V.....
PC	8.5	11.0 (212-323)N*..... FNSTPDAAN TLNLQAGGNTIN FNGIDGTGKLVLV
T2a	43.0	41.0 (212-618)T.....N*A.....G.....
PD	16.5	15.3 (324-473)N*..... VIQSAN AGGQVITFEHVDVGLGGTT
PH	44.0	42.0 (474-884) NNNALAAGSIQLD ENNGSVQLNHNTYLIT
T3b	30.0	(619-?)?.....N.T
T4c	23.0	19.9 (619-809)D.....N.T.....
T5a	22.0	(619-?)D.....N?T.....
T8	3.5	(810-?)	ISGPGNIVFNEIGNVA.....G
PA	3.2	5.3 (885-935)Q..... IANGQITGDKKNTIALSLGSDNSITV
T6	14.0	13.5 (885-1020)V.....Q.....
PBb	5.9	8.9 (936-1020) PNNPGTIYGLGLENGSPK
PF	25.0	24.2 (1021-1254) IVATQANKGTVTYLGNALVSNIG
T3a	33.0	(1021-?)S?.....Q.....A.....
T4a	26.0	24.2 (1021-1254)S?.....Q.....
PBa	5.2	12.0 (1255-1371) LLAKDPSDVATFVGA IATDTSAAVT
T7	5.2	12.0 (1255-1371)S.....I.....

^aFragment sizes expected from DNA sequence of *R. prowazekii* SPA gene and assuming strong homology of unknown *R. typhi* SPA gene sequence.

^bAmino acid residues which are identical to those deduced from the DNA sequence of the *R. prowazekii* SPA gene (shown as complete sequence). *R. prowazekii* CNBr sequences are shown above the DNA sequence data while *R. typhi* CNBr sequences are below. N* represents a modified amino acid.

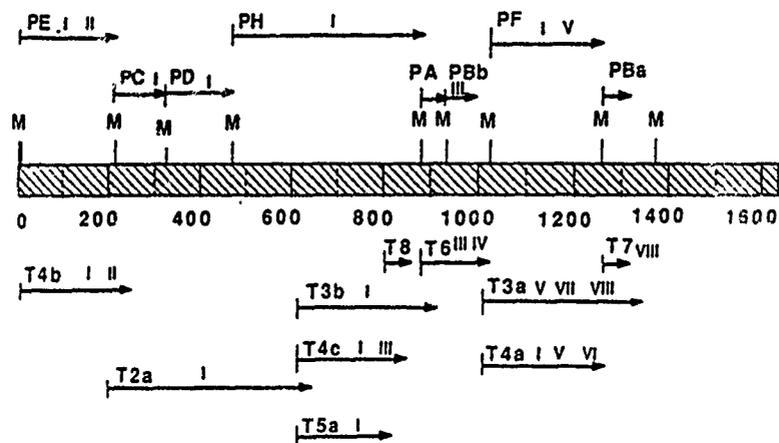


Fig. 2. Location of CNBr-derived fragments of SPAs from *R. prowazekii* and *R. typhi* identified by *N*-terminal amino acid sequencing (identified in Table 1 and Fig. 1) along the deduced amino acid sequence of the gene *spaP*. Arrow lengths of the CNBr fragments were determined by SDS-PAGE. M indicates the positions of methionines predicted from the *spaP* DNA sequence. Roman numerals correspond to the type of monoclonal antibodies reacting with each fragment (Table 3).

(Table 1). No intact SPA was recovered suggesting that quantitative CNBr cleavage was obtained. The sequences and estimated sizes permitted alignment of the fragments with the deduced amino acid sequence of the *R. prowazekii* SPA obtained from the *spaP* gene (Carl *et al.*, 1990) (Fig. 2, Table 1). Although the gene for the SPA of *R. typhi* has not yet been cloned and sequenced, the extensive homology between the sequences of the *R. typhi* CNBr fragments and regions encoded by the *spaP* gene permitted them also to be aligned (Fig. 2, Table 1). This tentative alignment was warranted given the extensive physical and immunological similarities between these two SPA proteins (Ching *et al.*, 1990, 1991). A total of sixteen CNBr fragments are predicted from the *spaP* gene sequences. The sequences and sizes, as well as the similar yields of 8 major *R. prowazekii* CNBr fragments (Fig. 1, Table 1) were in good agreement with that expected from the *spaP* sequence for amino acids 6–1371. However, none of the 7 CNBr fragments of 0.87–8.14 kDa expected from the *C*-terminus of *R. prowazekii* SPA (amino acids 1372–1612) were detected. Although some of these fragments as well as the expected *N*-terminal tetrapeptide fragment (amino acids 2–5) would be too small to be detected by PAGE, the three larger fragments with expected mol. wts ranging from 6 kDa to 8 kDa should have been detected.

Five CNBr fragments of *R. typhi* SPA were present in comparably large quantities (T4b, T2a, T4c, T6, T4a, Fig. 1) and had sequences and sizes expected to encompass amino acids 6–1254. Characterization of minor fragment T7 (corresponding to size and location to PBa, 1255–1371) completed identification of a full set of protein segments comparable to those found for *R. prowazekii* SPA. The minor fragment T3a appeared to encompass fragments T4a and T7, possibly due to incomplete cleavage at amino acid 1254. The presence of fragment T8 suggests that another methionine or acid labile site exists at 809. The minor fragment T3b may have resulted from incomplete cleavage at amino acid 809 and 884 and cleavage at an acid sensitive site within

T6. Large major fragments T1 and minor fragments T2b, PGa, and PGb (Fig. 1) were not sequenced successfully but appear to be incomplete cleavage products or possibly unresolved mixtures of several partial cleavage fragments as deduced from their reaction patterns with monoclonal antibodies (see below).

Two further observations support the view that predicted amino acids 1372–1612 are not present in the surface forms of *R. prowazekii* and *R. typhi* SPAs. The CNBr fragments closest to the *C*-terminus (T7 and PBa) were of 5.2 kDa size, which is less than half of the expected 12.0 kDa. Thus the *C*-terminal cleavage may actually occur within this fragment after translation of SPA mRNA. Further, the protease-derived "nicked" forms of both SPAs each lacked these fragments as well as PF, T3a, and T4a (see above, Fig. 1), all derived from the *C*-termini of the SPAs, indicating that the *C*-terminal region of both SPAs are particularly accessible to proteolytic attack. In contrast CNBr fragments derived from the *N*-termini of the SPAs were unaffected by digestion of native protein. Therefore, the discrepancy between the measured size of the SPAs and their size as deduced from the sequence of *spaP* arises from truncation of the SPAs at the *C*-termini rather than the *N*-termini.

In addition to the obvious differences in CNBr fragmentation patterns of *R. prowazekii* and *R. typhi* SPAs (Fig. 1), amino acid sequence variations were observed between the SPAs for all five homologous fragments analyzed (Table 1). T4c and T8, for which no comparable *R. prowazekii* fragments were obtained, also differed from the *spaP* sequence. Only two of the 14 amino acid differences detected in *R. typhi* SPA would be due to more than one base change in the *spaP* codons employed. Three amino acids of *R. prowazekii* SPA determined by direct peptide sequencing differed from the amino acids predicted from *spaP*. Amino acids 220 and 329 were expected to be asparagine. However, both PTH-amino acid derivatives eluted between the serine and glutamine derivative positions as an unknown

moiety with a peak height much larger than that expected for serine (data not shown). This moiety may be a modified asparagine. One example of naturally occurring asparagine modification, γ -*N*-methylasparagine, has been previously observed in allophycocyanin (Klotz *et al.*, 1986). The other predicted amino acid, lysine 894, was clearly detected as glutamine for both *R. typhi* and *R. prowazekii* by direct protein sequencing. Lysine 1028 was detected as glutamine again for *R. typhi* but for *R. prowazekii*, lysine was confirmed as the correct amino acid.

Epitope mapping of monoclonal antibodies

In order to determine which CNBr-derived fragments were being recognized by various SPA-specific monoclonal antibodies, we prepared blots with alternating lanes of CNBr fragments and pre-stained molecular weight standards. The blots were then cut into strips through the pre-stained molecular weight standards. Alternate strips were then immunodetected with monoclonal antibody or stained with Coomassie blue to identify the CNBr fragments immunodetected on the adjacent strip. By aligning these strips precisely using the bisected prestained standard, the assignment of reactive fragments was made less ambiguous (Fig. 3). Surprisingly, most of the immunodetection patterns were exceedingly complex, and only a few patterns permitted immediate identification of the reactive CNBr fragments (e.g. Fig. 3A P-15; Fig. 3B P-7). The 18 monoclonal antibodies tested (Table 2) could be subdivided into eight distinct patterns (Table 3). The sites recognized by the eight classes of monoclonal antibodies are also represented schematically (Fig. 2). The complexity observed in the reaction patterns appears to be due to two different factors. First, the immunoreactivity of some fragments was considerably greater than that expected from the intensity of the Coomassie blue stained gels or Western blots. Much of this reactivity was present in high mol. wt regions (PGa, PGb, T1 to T3b) where *N*-terminal sequencing was not successful. Many of these immunoreactive bands had no comparable Coomassie blue stainable band or the reaction patterns of different antibodies suggested several different fragments were present at indistinguishable positions. Therefore, this region appeared to contain small amounts of various fragments due to incomplete CNBr cleavage or cleavage at secondary acid labile sites. Consequently, these bands were ignored in the analysis, except as aids in grouping antibodies with similar reaction patterns. Alternatively, differences in the immunoreactivity of some fragments may merely reflect differences in the affinity of the monoclonal antibodies employed. The second factor contributing to the complex patterns is illustrated by Type I antibodies (P12 and P19, Table 3). Type I monoclonal antibodies reacted strongly with many clearly identified fragments on both *R. typhi* SPA (T2a, T3b, T4b, T4c, Fig. 3B) and *R. prowazekii* SPA (PC, PD, PE, PF, PH, Fig. 3A). Surprisingly, although all the other homologous fragments of the two SPAs showed comparable reactivity, T3a and T4a did not

Table 2. Characterization of anti-SPA monoclonal antibodies used to map binding sites on CNBr fragments of *R. typhi* and *R. prowazekii* SPA

Monoclonal antibody (clone designation)	Immunoglobulin subclass	SPA ELISA specificity ^a	Natural fragment pattern ^b
P6 (P53-5E12.1)	KG _{2b}	P>>T	A
P7 (P51-4B12.1)	KG ₁	P>>T	A
P8 (P53-2G12.2)	KG ₁	P>>T	A
P9 (P51-4D8.1)	KG _{2a}	T = P = C	B
P10 (P53-3D1.1)	KG ₁	T = P = C	B
P12 (P46-2G4.1)	KG _{2b}	T = P = C	A
P14 (PT47-1C5.1)	KG ₁	T = P	B
P15 (P46-3D3.1)	KG ₁	T = P	B
P16 (P51-5C12.2)	KG ₁	T = P	B
P18 (P53-2D7.1)	KG _{2b}	T = P = C	B
P19 (P53-3D12.2)	KG ₁	T = P = C	A
T6 (T66-1C10.1)	KG _{2a}	T>>P	B
T7 (T66-1E8.1)	KG ₁	T	B
T9 (T22-3E6.1)	KG ₁	T	B
T10 (T28-2F5.1)	KG ₁	T	B
T13 (T66-2E8.1)	KG ₁	T = P	B
T15 (T66-2D3.1)	KG ₁	T = P	B
T16 (T71-3G11.1)	KG ₁	T = P = C	B

^aP = *R. prowazekii*, T = *R. typhi*, C = *R. canada*.

^bPattern of major fragments of SPA detected by Western blotting.

react like PF, suggesting that the *R. typhi* SPA contained a different sequence in this region. We hypothesized that Type I antibodies might recognize either a recurrent post-translational modification such as glycosylation or a repeating sequence unit. Therefore, we treated the CNBr fragments with trifluoromethanesulfonic acid to remove potential glycosyl groups (Edge *et al.*, 1981) and then compared the polyacrylamide gel patterns and immunoblots of treated and untreated samples. No difference was found (data not shown). Alternatively, Type I monoclonal antibodies might recognize small

Table 3. Monoclonal antibody classification according to reaction with CNBr fragments of *R. prowazekii* and *R. typhi* SPA

Type	Monoclonal antibodies	Reaction with CNBr fragment	
		PSPA	TSPA
I	P12, P19	PE	T4b
		PC	T2a
		PD	T3b
		PH	T4c
		PF	
II	P6, P7, P8	PE	T4b
III	P9, P10, P18	PBb	T4c
			T6
IV	T6	—	T6
V	P15, T15	PF	T3a
			T4a
VI	T9, T10	—	T4a
VII	P14, P16, T16, T13	—	T3a
VIII	T7	—	T3a
			T7

regions of repetitive structural motifs which seem to be scattered over much of the SPA molecule (Dasch *et al.*, 1991). One of these repeating motifs is illustrated in Fig. 4. Strong homology exists between fragment PE and PC (Fig. 4, amino acid 103-120 and amino acid 218-235). The sequence in this region was also confirmed by amino acid sequencing of fragment PC and homologous fragment T2a from *R. typhi* SPA. A region with a lower degree of similarity is also present in fragment PD, PF and PH (Fig. 4). It is perhaps noteworthy that both of the modified asparagines detected by sequencing are also present in this region although they are not in the same alignment. Similarly, T2a also contains modified

asparagine at the same position as found in PC. In the position aligned with amino acid 329 of PD, the comparable asparagine in PC and T2a was not altered as detected by direct sequencing (Table 1, Fig. 4). It is possible that the asparagines in PE, PH, and PF aligned at this position may also be modified but they have not been examined yet by direct sequencing. Type III monoclonal antibodies, like Type I monoclonal antibodies, also showed strong binding to more than one fragment of *R. typhi* SPA (fragment T6 and T4c, antibody P10, Fig. 3B) but very weakly, if not at all, to T3b and only to one of the homologous regions in *R. prowazekii* SPA (PBb but not PH, Fig. 3A).

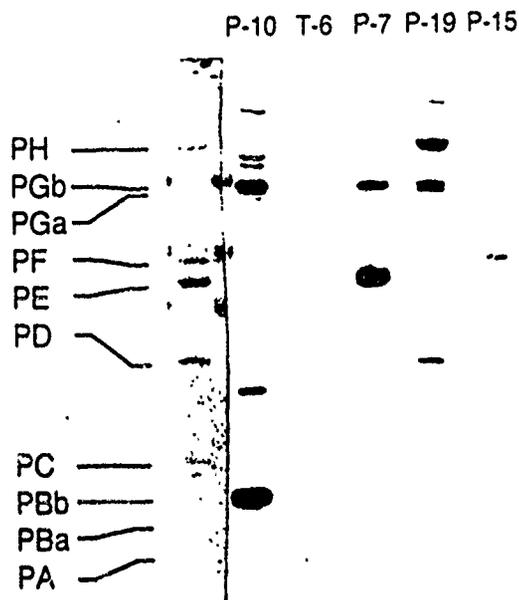


Fig. 3(A)

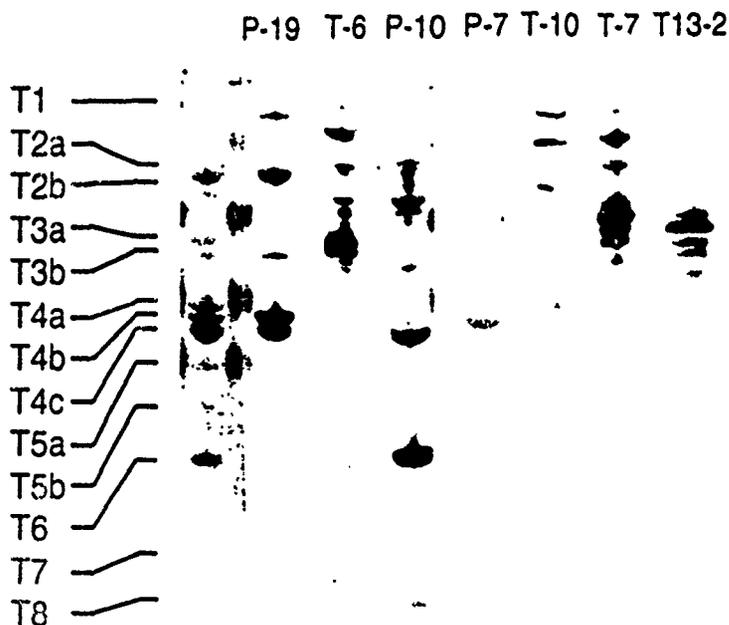


Fig. 3(B)

Fig. 3. Examples of monoclonal antibody epitope mapping on CNBr-derived fragments of SPA from *R. prowazekii* (A) and SPA from *R. typhi* (B). In each panel the first strip was stained with Coomassie blue to identify the location of each fragment indicated (with the aid of the prestained molecular weight standards found on both edges of all the strips). The other strips were separately immunostained with the indicated monoclonal antibody.

PE (103-120)	T	G	Q	G	I	T	V	Q	E	A	S	N	T	I	N	A	Q	N
PC (218-235)	A	A	N*	T	L	N	L	Q	A	G	G	N	T	I	N	F	N	G
PH (624-641)	V	Q	L	N	H	N	T	Y	L	I	T	K	T	I	N	A	A	N
PF (1112-1129)	T	S	T	W	G	D	N	T	S	I	S	T	T	L	N	V	S	S
PD (315-332)	S	G	Q	V	Y	A	K	N	M	V	I	Q	S	A	N*	A	G	G

Fig. 4. Comparison of structurally similar amino acid sequences found on different CNBr fragments of *R. prowazekii* SPA recognized by type I monoclonal antibodies. Amino acids with identity or conserved substitutions are boxed. Categories of conservative substitutions are as follows: S, T, P, A, and G; N, D, E, and Q; H, R, and K; I, L, V, F, and Y. Sequences confirmed by direct peptide sequencing are indicated (•) while the others are inferred from the sequence of *spaP*. N* is a modified amino acid.

In contrast to these multiple reactivities, the other six classes of antibodies reacted predominantly with only one clearly identified major CNBr fragment (Table 3, Fig. 2). The precise binding site(s) of Type VII antibodies were best defined. Fragment T3a and T4a have the same *N*-termini. Fragment T3a, which is approximately 8 kDa larger than fragment T4a appears to encompass T4a and T7 due to an incomplete cleavage at Met 1254. Type VII antibodies do not react with fragment PF, PEa, T4a, or T7, but do react very strongly with fragment T3a (antibody T13, Fig. 3B), indicating that the binding site must be very close to the cleavage site at Met 1254. Similarly, Type V antibodies bound to PF (antibody P15, Fig. 3A) and to both T4a and T3a but not to T7, suggesting that type V antibodies bound to a different site closer to the *N*-terminus than did type VII antibodies. Three different classes of monoclonal antibodies reacting preferentially with *R. typhi* SPA (Table 2) could be defined (Types IV, VI, and VIII). Type VI binds somewhat weakly to fragment T4a but not at all to T3a (antibody T10, Fig. 3B) possibly because of the small quantity of the latter fragment. Because of the poor binding to T4a relative to larger fragments, it is likely that the binding site for this antibody includes some of the *C*-terminus of T6 as well as the amino terminus of T4a. The other T specific site pattern (Type VIII) is clearly different from Type VI and appears to be present on the most *C*-terminal fragment T7 (antibody T7, Fig. 3B). Type IV antibody bound to fragment T6 and apparently to T3b which is a partial cleavage fragment containing fragment T6 (antibody T6, Fig. 3A, B). Finally Type II antibodies, which react preferentially with *R. prowazekii* SPA, bound well to the *N*-terminal fragment PE and rather weakly to the homologous fragment T4b of *R. typhi* SPA (antibody P7, Fig. 3A, B).

DISCUSSION

Earlier attempts to map the epitopes of SPAs by Western blotting following complete protease digestion and PAGE separation of the fragments were not successful. The very β -structure-rich SPAs become quite insoluble and extremely sticky after denaturation. High concns of chaotropic reagents are required to keep these

proteins in solution, and under conditions such as 4 M urea, 2% SDS and 1% Triton X-100, or 3 M guanidine, we have been unable to find a protease which permits complete digestion in reasonable yields. Separation of mixtures of protease- or CNBr-generated fragments of SPA by various HPLC methods was also not very successful. The separation of CNBr-generated fragments by the special SDS-PAGE system and subsequent recovery on PVDF membrane following electroblotting is the best approach yet developed for mapping the binding sites of monoclonal antibodies with rickettsia-derived protein. The present epitope mapping study has permitted us to identify the binding sites of six types of antibodies to five different CNBr fragments, which range in size from 5 to 36 kDa. The larger fragments still pose significant problems for further identification of the epitopes involved in the antibody binding. HPLC analysis of protease digests of individual purified small CNBr fragments will likely have adequate resolution for the direct identification of smaller peptides with binding activity. However, it appears likely that other chemical cleavage methods may be necessary to further reduce the size of the large CNBr fragments of SPA before they can be analyzed.

Although mapping of epitopes in which modified amino acids may be important in the antibody binding (e.g. Type I and III antibodies) must necessarily be done with rickettsia-derived SPA, refined mapping of the other six types of apparently linear epitopes may be possible by synthesis of continuous and overlapping short peptides (Geysen *et al.*, 1987). The present studies suggest that synthesis of peptides restricted to the *N*-terminal region [1-211 amino acids) and the truncated *C*-terminal region (amino acids 885-1371) will permit precise mapping of these epitopes. However, this time-consuming and expensive approach will first require cloning and sequencing of the *R. typhi spaT* gene since three of the types of monoclonal antibodies available are relatively specific for *R. typhi* SPA.

CNBr fragments corresponding to amino acids 6 through 1371 were identified for both *R. typhi* and *R. prowazekii* SPAs. The SPAs behave aberrantly in different physical environments so that molecular weights of 85-135 kDa have been obtained by different methods

(Ching *et al.*, 1991). The open reading frame of *spaP* encodes a protein of 169 kDa (Carl *et al.*, 1990). The molecular weights for CNBr fragments of SPA were estimated on SDS-PAGE. In general, the results for larger fragments (14–43 kDa) are only slightly higher than the expected values. For smaller fragments (3.2–8.5 kDa), the estimated values are 60–77% of those predicted. Since small fragments PBa and T7 are only 43% of the size expected, the actual C-terminus of both SPAs is probably closer to amino acid 1326 [70% of 12 kDa = 8.4 kDa: $(5.2/8.4) \times 117$ amino acids + 1254 = 1326] than 1371. The C-terminus appears to be particularly sensitive to protease digestion since the protease "nicked" forms of SPA have a slightly decreased mol. wt and C-terminal CNBr fragments PBa, T7, PF, and T4a disappear or are greatly reduced in yield relative to intact SPA. In a separate experiment by line blotting (Raoult and Dasch, 1989), as expected nicked SPAs had reduced reactivity with monoclonal antibodies binding to these fragments.

The SPAs do not appear to undergo significant N-terminal processing since CNBr fragments expected at amino acid 6 were recovered from both species. Furthermore, Edman degradation of uncleaved *R. typhi* SPA gave the sequence ?VM corresponding to amino acids 3–5 of *spaP* (Ching *et al.*, 1990, 1991) suggesting that at most 2 amino acids had been removed. The expected tetrapeptide generated by CNBr treatment was not detected on the gel presumably because it was too small. In contrast, the N-terminus of *R. prowazekii* SPA was not accessible for Edman degradation (Ching *et al.*, 1990), possibly due to a fatty acid modification. Selected subsets of eucaryotic cellular proteins and viral polypeptides have long fatty acids attached to their N-termini (Towler and Gordon, 1988). The N-terminal sequence of MGAAM predicted from gene *spaP* is of particular interest because both polioma virus VP2 and SV40 virus have the same sequence GAA and both are myristoylated at the N-terminal glycine (Streuli and Griffin, 1987). Since rickettsiae grow in the cytoplasm of the eucaryotic cell and the SPA is surface exposed, it may be a target for host cell modification. Whether the variation in N-terminal sequences of the two species may account for the apparent difference in modification is not known.

Since the SPA is not significantly truncated at the N-terminus, the smaller size of the intact mature protein as compared to that deduced from the *spaP* gene sequence together with the lack of the expected C-terminal CNBr fragments suggest that about 250 amino acids are either removed after SPA synthesis is complete, or that the genetic information is not transcribed or translated. However, the partial sequence of the 120 kDa SPA gene of *R. rickettsii* (Carl *et al.*, 1990; Gilmore *et al.*, 1989) is more highly homologous to *spaP* in the "missing region" than in the rest of the protein. From amino acid 1331 to 1356 of both proteins, which is just before the presumed membrane spanning α -helix, there are nine conserved sites for tryptic and chymotryptic cleavage. Among these nine sites, five of them are also conserved in the sequence

of the 190 kDa SPA gene of *R. rickettsii* (Anderson *et al.*, 1990). This is the only area where so many proteolytic sites are clustered in such a short segment of sequence. These observations all suggest an important role for this region in the processing and translocation of these proteins. It is possible that the small amounts of reactive *R. typhi* and *R. prowazekii* SPA bands with sizes greater than the major 120 kDa band detected on Western blots with anti-SPA monoclonal antibodies (Ching *et al.*, 1991; Dobson *et al.*, 1991) are the unprocessed full length translation products. Indeed the recombinant *R. prowazekii* SPA product made in *E. coli* is larger than the mature SPA obtained from the rickettsiae (Dobson *et al.*, 1991). Synthetic peptides corresponding to amino acids present in the predicted C-terminus of *spaP* have been prepared in order to make specific antibodies that may react with the unprocessed *R. prowazekii* SPA.

Type I antibodies reacted with many different major CNBr fragments of both SPAs. Deglycosylation of these fragments did not lead to a shift in electrophoretic mobility or a change in the recognition of these fragments by antibodies as determined by immunoblotting. Total sugar analyses of the HPLC purified SPA of *R. prowazekii* indicated that there was less than one sugar per SPA molecule (Ching *et al.*, 1991). Therefore, repeated sugar groups could not explain the recognition of multiple fragments by a single antibody. Alternatively, we hypothesized that repeating structural units might be scattered along the SPA molecule even though no large repetitive elements like those comprising 40% of the *R. rickettsii* 190 kDa SPA (Anderson *et al.*, 1990) are found in *spaP* (Carl *et al.*, 1990). A stretch of amino acid which bears some sequence homology was found in all the fragments which react with type I antibody (Fig. 4). A detailed comparison study of this gene sequence with that of *spaP* and the *R. rickettsii* 120 kDa gene revealed that different types of small repeating motifs homologous to small portions of the 190 kDa repeat sequences are scattered over the much of both the *R. prowazekii* and *R. rickettsii* 120 kDa SPAs but in different locations (Dasch *et al.*, 1991). The motif shown in Fig. 4 is only one example of the complex repetitive motifs present in these proteins. Although the sequences are not completely conserved, the conservative amino acid changes in these motifs may form similar three dimensional structures which may account for the multiple antibody reactivities observed. The presence of repetitive epitopes on the surface of *R. prowazekii* was also suspected by McDade *et al.* (1985) when they observed a biphasic titration curve for several toxin-neutralizing monoclonal antibodies (McDade *et al.*, 1985). It is of interest to note that the modified amino acids N* 220 and N* 329 are also within one of these repetitive motifs. Whether the antibodies with multiple reactivity recognize these modified asparagines or possibly other modified lysine residues (Table 1) or whether their reactivity could result from the binding to domains with similar charge clusters or hydrophobicity characteristics is unknown. If these antibodies do not bind to a modified amino acid, it may be possible to inhibit antibody binding to these

hypothesized repeat elements with a single synthetic peptide. This possibility is being investigated currently.

The best localized antibody binding epitope is that which is recognized by type VII antibody. As previously mentioned, this type of antibody reacts with fragment T3a which is generated from the incomplete cleavage at methionine 1254. No antibody was found to react with fragments PA, PBa and T8, with estimated molecular weights of 3.2, 5.2, and 3.5 kDa, respectively. We do not know whether this lack of recognition by antibody is due to the absence of epitopes on these fragments. Antibodies raised to synthetic peptides corresponding to these sequences may provide more direct evidence about their immunogenicity.

SPAs are the immunodominant antigens of typhus rickettsiae. Once the domains involved in their antigenicity are defined, they can be used to design species-specific reagents useful for serodiagnosis. Current methods for serodiagnosis of rickettsial diseases require antigen purification from infected yolk sacs of embryonated chicken eggs or from infected cells. The identification of antigenic peptides may permit the preparation of a new generation of widely available inexpensive reagents for the diagnosis of epidemic and endemic typhus. Furthermore, precise mapping of the B and T cell epitopes present on the SPAs which are recognized by the human immune system may permit the design of highly immunogenic synthetic vaccines for these rickettsiae.

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