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A Pneumococcal Surface Protein (PspB) That Exhibits the Same Protease Sensitivity as Streptococcal R Antigen

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A monoclonal antibody against *Streptococcus pneumoniae*, designated T4A49, detected a 64,000-molecular-weight protein that was immunologically unrelated to the previously described surface protein PspA. This new protein, designated PspB, was pepsin sensitive and trypsin resistant, as is the R antigen of type 28 group A streptococci.

Pneumococcal surface protein A (PspA) plays a role in the virulence of pneumococci and can elicit protective antipneumococcal antibodies (9–12). This observation led us to investigate other surface proteins which may be imunogenic and have diagnostic or vaccine potential. This article describes an antibody produced by hybridoma cell line T4A49 that reacts with a surface protein on *Streptococcus pneumoniae* EF-3296. This protein, like the R antigen of group A and group B streptococci, is susceptible to pepsin and resistant to trypsin digestion. The protein R antigen on group A streptococci was described in 1952 by Lancefield and Perlmann (5). It was believed that the R antigen was unrelated to virulence because anti-R antibodies failed to protect mice against infection by group A streptococci (5). However, the R antigen has been suggested to be important for virulence of type II group B streptococci (7), and anti-R antibodies have been reported to protect mice against infection by type II group B streptococci (6).

Production of monoclonal antibody. X-linked immunodeficient CBA/N male mice (Dublin Laboratories, Dominion, Va.) were immunized with four weekly intravenous injections of 5×10^8 heat-killed type 4 pneumococci, strain EF-3296 (obtained courtesy of Catharina Svanborg-Eden, University of Goteborg, Sweden). Three days after the last injection, spleen cells were fused with the non-immunoglobulin-producing mouse myeloma line P3-X63-Ag.8.653 (4) as described previously (9). A single hybrid, designated T4A49, produced antibody that reacted with EF-3296, the immunizing pneumococcal strain, but failed to react with phosphocholine, a highly immunogenic determinant common to the cell walls of all pneumococci (1, 2). T4A49 ($\gamma_{2a}\kappa$) was recloned, expanded, and used to produce ascites in (CBA/N $\times$ BALB/cJ)F_1 mice.

Strain distribution of epitope. T4A49 antibody isolated from mouse ascites fluid by precipitation with 48% saturated ammonium sulfate was ^{125}I labeled by a chloramine-T procedure (3). The labeled antibody was assayed for its ability to bind to 5×10^7 CFU of viable encapsulated pneumococci as previously described (9). Table 1 shows that labeled T4A49 reacted with 6 of 14 type 4 isolates and 3 of 14 isolates of 11 other capsular types (pneumococcal isolates were obtained from C. Svanborg-Eden, and from Barry Gray and Hugh Dillon in the Department of Pediatrics, University of Alabama at Birmingham). The immunoglobulin M antiphosphocholine hybridoma 22.1A4, included as a control, showed a range of 12 to 60% binding to all pneumococcal isolates tested.

The variations in the reactivity of T4A49 with different strains suggest that monoclonal antibodies to this protein, like antibodies to PspA, may be useful for distinguishing different isolates of the same capsular type (11, 12).

The failure of T4A49 to react with all strains of pneumococci may not be because the strains lack PspB as detected by T4A49, but rather because they express an antigenic variant of the protein. A similar situation has been observed for PspA, for which variants are known that are not detected by existing monoclonal antibodies but react with a heterologous rabbit antiserum to PspA (M. J. Crain, D. F. Talkington, and D. E. Briles, manuscript in preparation).

Protease sensitivity. Treating EF-3296 with trypsin (9) slightly increased its ability to bind T4A49. Treatment with pepsin (9) reduced the ability of EF-3296 to bind T4A49 by greater than 95%. This is the same pattern of protease sensitivity exhibited by the R antigen of type 28 group A streptococci (5). In contrast, PspA is susceptible to both trypsin and pepsin (9).

To test pneumococcal cell wall extract (CWE) (10) for the presence of antigen detected by T4A49, 10 μl of threefold serially diluted samples was spotted onto a nitrocellulose membrane and developed as a dot blot (10) with T4A49 tissue culture supernatant. Treating CWE with pepsin at pH 2 for 3 h gave a reciprocal endpoint dilution of <3, whereas incubation of CWE at pH 2 without pepsin, or with trypsin at pH 7, resulted in an endpoint of 1/243. The endpoint of untreated CWE was 1/729. These results, and those with protease treatment of whole bacteria, make it likely that the epitope detected by T4A49 is a protein that is relatively insensitive to trypsin.

Surface location of epitope. To confirm the surface location of PspB, we carried out immunofluorescence staining of viable encapsulated pneumococci with T4A49. Exponentially growing pneumococci were harvested, washed, and suspended at 10^8 cells in 100 μl of phosphate-buffered saline (PBS) (pH 7.2). The cells were incubated with T4A49 (10 μg/ml) on ice for 20 min. After being washed and resuspended in 100 μl of PBS, the pneumococci were incubated with secondary antibody, fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G2a antibody, for 20 min on ice. After being washed, the cells were suspended in 500 μl of PBS and 100 μl was spread on a microscope slide and allowed to air dry. The cells were gently heat fixed to anchor them to the slide, covered with a cover slip, and viewed with a fluorescence microscope. There was agreement between

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TABLE 1. Binding of T4A49 to pneumococcal isolates

Capsular serotype[a]	No. of isolates	No. of isolates according to % binding		
		<3%[b]	3–30%	>30%
4	14	8	2	4
1	1	1	0	0
2	1	1	0	0
3	2	2	0	0
5	2	2	0	0
6	1	1	0	0
8	1	0	1	0
9	2	0	1	1
10	1	1	0	0
14	1	1	0	0
19	1	1	0	0
23	1	1	0	0

[a] Danish typing system.
[b] Percentage of binding of 2×10^4 cpm of ^{125}I-labeled T4A49 after incubation with 5×10^7 CFU of viable encapsulated pneumococci at 37°C for 30 min.

those isolates that bound ^{125}I-labeled T4A49 and those that stained in the immunofluorescence assay. The cells exhibited a uniform pattern of staining (not shown), indicating that the binding was not the result of reactions of the antibody with an occasional damaged bacterium.

A similar fluorescence procedure has been used to detect streptococcal M protein on the surface of bacteria (13). However, in our study, we stained viable pneumococci in suspension before fixing the bacteria to a microscope slide to ensure that accessibility of the epitope was not dependent on the fixation process.

Size of PspB. To estimate the size of the molecule that reacted with T4A49, CWE prepared from different pneumococcal strains were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then immunoblotted as previously described (10). The EF-3296 protein detected by T4A49 exhibited a molecular weight of 64,000 (Fig. 1). No apparent variation in the molecular weight of the antigen was observed among four strains. Those strains that failed to bind T4A49 in the binding assay showed no reaction in the immunoblot.

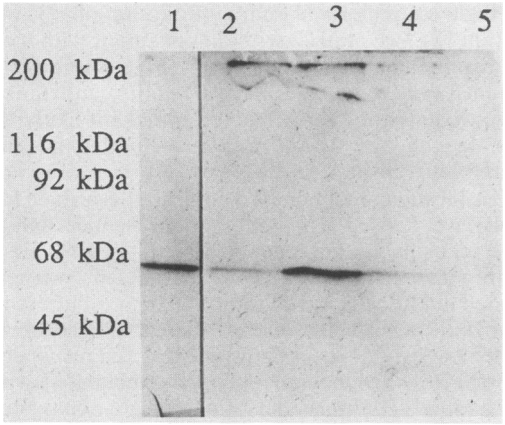

FIG. 1. Immunoblot of CWE from pneumococcal isolates for the presence of PspB. Lane 1, BG-7428 type 9; lane 2, EF-8594 type 4; lane 3, EF-3296 type 4 (the strain against which T4A49 was made); lane 4, EF-4847 type 8; lane 5, EF-7908 type 4 (an isolate that does not react with T4A49). The blotted antigens were reacted with T4A49 to detect PspB. kDa, Kilodaltons.

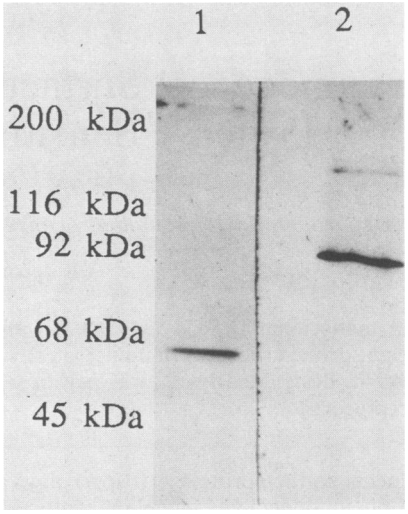

FIG. 2. Immunoblot of CWE from strain BG-7428 type 9 which reacts with both T4A49 and Xi126, a monoclonal antibody against PspA. After the antigens were blotted to nitrocellulose, the membrane was cut and lane 1 was reacted with T4A49, to detect PspB (64 kilodaltons [kDa]), and lane 2 was reacted with Xi126, to detect PspA (86 kilodaltons).

To determine if T4A49 was detecting epitopes on PspA, we immunoblotted CWE prepared from a pneumococcal strain known to react with both T4A49 and the anti-PspA monoclonal antibody Xi126. Pneumococcal strain BG-7428 type 9 has been demonstrated to react with both T4A49 and Xi126 (W. D. Waltman II, personal communication). The molecular weight of the protein detected by T4A49 in strain BG-7428 was different from that of PspA detected by Xi126 (Fig. 2). Thus, we have designated the protein(s) detected by T4A49 as PspB.

Inability of T4A49 to protect mice. CBA/N mice were injected intraperitoneally with 7, 70, or 700 μg of T4A49 1 h prior to intravenous infection (8, 9) with approximately 100 50% lethal doses of EF-3296 (the 50% lethal dose of EF-3296 for XID mice is <10). No evidence of protection was observed. In the case of PspA, we observed that only two of the four antibodies we produced to different epitopes of PspA were protective against pneumococcal infection (9). Thus, our observation with a single anti-PspB antibody does not rule out the possibility of protective effects with other antibodies to this antigen.

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