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Penicillin-Binding Proteins of Penicillin-Susceptible and -Resistant Pneumococci: Immunological Relatedness of Altered Proteins and Changes in Peptides Carrying the β-Lactam Binding Site

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There are several major differences between the penicillin-binding proteins (PBPs) of highly penicillin-resistant and -susceptible strains of pneumococci. The highest-molecular-size PBP 1a (98 kilodaltons [kDa]) of susceptible pneumococci is not detectable in resistant bacteria. Instead, resistant strains contain a PBP of smaller size: 92 and 94 kDa in South African strains 8249 and A95210, respectively, and 96 kDa in New Guinea strain 2955 (S. Zighelboim and A. Tomasz, Antimicrob. Agents Chemother. 17:434–442, 1980). Using antibodies prepared against PBP 1a of penicillin-susceptible pneumococci, we demonstrated that these anomalous-sized proteins in the resistant strains are immunologically related to PBP 1a of penicillin-susceptible bacteria. A second difference between the PBP patterns of strain 8249 and the susceptible pneumococci is that the 78-kDa PBP 2b is not detectable by the radioactive penicillin binding assay in the resistant strain. Using antibodies prepared against PBP 2b of susceptible cells, we demonstrated the presence of PBP 2b in membrane preparations from strain 8249 cells. Thus, the poor detection of this PBP appears to be related to its greatly decreased affinity for the antibiotic molecule. We also compared the patterns of penicillin-labeled peptides derived from PBPs of resistant and susceptible cells during partial proteolysis by V8 protease. Several changes were observable in small peptides carrying the beta-lactam binding site generated from the high M_r (PBP 1a-related) binding proteins. In contrast, no differences in the pattern of penicillin-labeled peptides were seen when the pattern of PBP 2a of susceptible pneumococci was compared with the peptide pattern of PBP 2a from resistant strains. One of the resistant isolates (strain 2955) also had a PBP 3 with a higher-than-normal molecular weight. This protein gave strong positive reaction with antibodies against PBP 3 of susceptible cells. Examination of the pattern of penicilloyl peptides generated from the susceptible and resistant PBP 3s during partial proteolysis revealed only differences which seem to reside distant from the beta-lactam binding site.

Each species of bacteria examined so far contains at least one and usually several proteins capable of binding penicillin (penicillin-binding proteins [PBPs]) detectable by the simple electrophoretic-fluorographic technique (19). Several of these PBPs have been shown to be cell wall synthetic enzymes, and individual PBPs of a bacterium are unique proteins distinguishable by genetic, immunological, and structural criteria (for a review, see reference 22). The number, molecular size, and antibiotic binding capacity of PBPs appear to be highly conserved within a species so long as isolates of similar penicillin susceptibilities are compared. On the other hand, PBPs with altered properties have been identified in bacterial isolates with increased resistance to penicillin or other beta-lactam antibiotics.

A variety of PBP alterations have been associated with this type of resistance. In methicillin-resistant *Staphylococcus aureus* (2, 8, 13, 26) or *Streptococcus faecium* (7), resistance is related to the acquisition of a new PBP recognizable by its distinct molecular size and low reactivity toward the antibiotic. In gonococci (4), *Haemophilus influenzae* (16), and the isolates of pneumococci with low- or intermediate-level resistance (10, 11, 17), resistant bacteria seem to retain a normal set of PBPs, but one or more of these appear to have a lower binding capacity for the antibiotic than the same PBPs from susceptible cells. In comparison, the two highly penicillin-resistant South African strains of pneumococci previously studied (27) contain much more complex alterations: they have some PBPs of unchanged molecular size with a lower capacity for penicillin binding; at least two of the PBPs (1a and 2b) characteristic of susceptible cells are not detectable, and resistant cells contain an additional PBP of unique molecular size (92 kilodaltons [kDa]; PBP 1c) not seen in susceptible bacteria. Analysis of genetic transformants with low and intermediate levels of resistance revealed that PBP 1c appeared only at and above a distinct level of resistance and simultaneously with the disappearance of PBP 1a. In fact, PBPs 1a and 1c would never be detected in the same cell (20). Recently, a PBP 3 of higher-than-normal molecular size was also detected in one penicillin-resistant isolate (11). The identification of PBPs is usually based on the formation of covalent complexes between radioactive benzylpenicillin (the only labeled beta-lactam which is commercially available) and these proteins, and it is conceivable that the affinity for penicillin becomes so low as to make detection of a PBP by the binding assay impossible. In addition, a greatly increased rate of deacylation of the penicilloyl PBP could result in poor detection of a binding protein by the fluorographic technique. The recent isolation of antibodies against purified pneumococcal PBPs (T. Briese, Ph.D. dissertation, Freie Universität, Berlin, Federal Republic of Germany, 1986; H. Ellerbrok, Ph.D. dissertation, Freie Universität, Berlin, Federal Republic of Germany, 1986) provided a new method for the analysis of the PBPs of resistant cells that is not dependent on the

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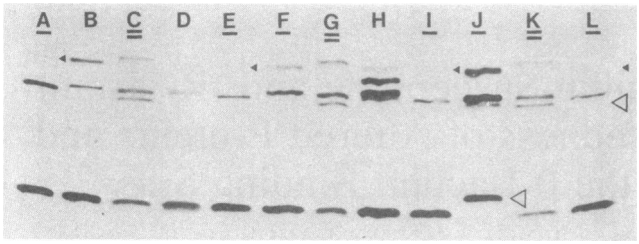

FIG. 1. PBP profiles of penicillin-susceptible and -resistant strains of pneumococci. The PBP patterns of strains used in this study are indicated by underlining the capital letter designation of the particular lane on the gel. A single underline (lanes A, E, F, I, J, and L) represents resistant strains examined in this study. Underlining in double (lanes C, G, and K) represents the susceptible R6 strain. The arrangement of the various membrane preparations on the single gel was such that comparison of electrophoretic mobilities would not be too difficult. For this reason, the most important strains (8249 and R6) were run in more than one lane. PBPs of penicillin-susceptible laboratory reference strain R6 (lanes C, G, and K) and of the three penicillin-resistant strains used in this study, i.e., strains 8249 (lanes A, E, I, and L), A95210 (lane F), and P2955 (lane J), were run on the same gel after a common exposure to 0.2 μg of [^{3}H]penicillin per ml at 37°C for 10 min, as described in Materials and Methods. Also run on the same gel were the PBPs of two additional penicillin-resistant strains, 140 (lane B; MIC ≃ 0.5 μg/ml) and A9229 (lane D; MIC ≃ 1.0 μg/ml), which, similar to strain 8249, contain a high-molecular-size PBP 1c (about 92 kDa) in place of PBP 1a of the susceptible pneumococci. In lane H, the PBPs of a *Streptococcus mitis* strain (P111) are shown. These bacteria also contain a 92-kDa high-molecular-size PBP which was used here simply as a position marker. Because of the low affinity for penicillin, PBP 1c of strain 8249 showed only weak labeling (solid arrowheads). Also note the lack of detectable PBP 2b (e.g., lane L, empty arrowhead). Strain P2955 (lane J) has an anomalous (52-kDa) PBP 3 (empty arrowhead).

formation of acylated proteins. We applied this method for the determination of the relatedness of the PBPs of susceptible and resistant isolates in cases in which the technique of penicillin binding detected differences or produced ambiguous results. Furthermore, the PBP-peptide pattern produced by V8 protease was analyzed to gain some information about the nature of alterations in the PBPs of resistant strains.

MATERIALS AND METHODS

Strains and growth conditions. Penicillin-susceptible *Streptococcus pneumoniae* R6 is a derivative of unencapsulated Rockefeller University strain R36A which has a benzylpenicillin MIC of 0.006 μg/ml. Strains 8249 and A95210 are penicillin-resistant clinical isolates with MICs of 6 and 2 μg/ml obtained from H. Koornhof of Johannesburg, South Africa. Strain 2955 is a clinical isolate (MIC, 1 μg/ml) from Papua, New Guinea, obtained by D. Hansman. Strain pen 6 was constructed by genetic transformation using DNA from strain 8249 and R6 as the recipient. This transformant has the same penicillin susceptibility and the same PBP profile as DNA donor strain 8249, and membranes prepared from this tranformant were used interchangeably with membranes from strain 8249 in all the PBP experiments described here. Details of the genetic transformation and the selection procedures were previously reported (27). Cultures were grown in a casein hydrolysate medium (14) supplemented with yeast extract (0.2% [wt/vol] final concentration; Difco Laboratories, Detroit, Mich.).

Membrane preparation. Cells were recovered by centrifugation (4,000 × *g* for 10 min). The cells were washed and suspended in 10 mM potassium phosphate buffer (pH 8.0) and broken by agitation with 100-μm glass beads in a Mickle apparatus (Gomshall, Surrey, England) for 2 h. Unbroken cells and cell walls were removed by centrifugation (27,000 × *g* for 10 min).

Membranes were recovered by ultracentrifugation (200,000 × *g* for 90 min), washed, and suspended in 10 mM potassium phosphate buffer (pH 8.0). All steps in the preparative procedure were performed at 4°C. Protein concentration was determined by the method of Lowry et al. (15) using bovine serum albumin as the standard. The membranes were stored at a concentration of 50 mg/ml at −70°C until use.

PBP labeling. Samples of membrane suspensions containing 150 to 200 μg of protein were diluted to a final volume of 100 μl in 50 mM potassium phosphate buffer, labeled with [^{3}H]penicillin, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by fluorography, by a previously published procedure (23–25). When PBPs were labeled with [^{3}H]proprionylampicillin ([^{3}H]PA), a previously described procedure (6) was followed. [^{3}H]benzylpenicillin (ethylpiperidinium salt) with a specific activity of 27 Ci/mmol was provided by Merck & Co., Inc., Rahway, N.J., and [^{3}H]PA was synthesized as previously described (18). These two reagents label the same PBPs in pneumococci.

Partial proteolysis with V8 protease. After a dimension 1 SDS-PAGE, the lanes were cut and layered on top of a dimension 2 15% SDS-PAGE gel. V8 protease was included in the stacking gel (3) at a final concentration of 0.2 μg/ml, and electrophoresis was done as described above. This protease hydrolyses peptide bonds at glutamic acid residues.

Preparation of antibodies against PBPs. Purified prepara-

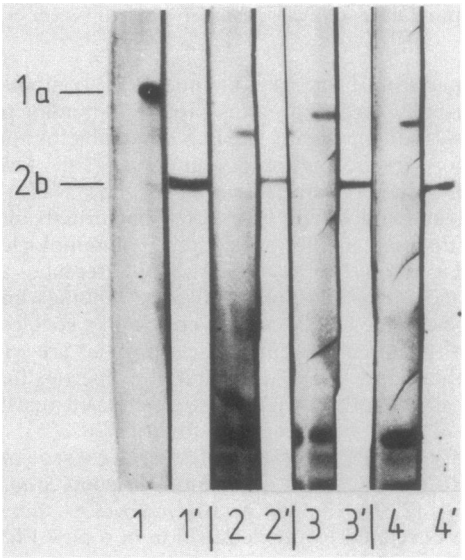

FIG. 2. Immunoblots of membrane proteins from penicillin-susceptible and -resistant strains using anti-PBP 1a and anti-PBP 2b antisera. Membrane proteins (100 μg) of the various strains were separated on SDS-PAGE and blotted onto nitrocellulose paper. Each lane was cut in two parts, and one was incubated with antiserum against PBP 1a (lanes 1 to 4) and the other was incubated with anti-PBP 2b antiserum (lanes 1′ to 4′). The immunoblot was developed as described in the text. Lanes: 1 and 1′, R6 (penicillin-susceptible strain); 2 and 2′, strain 8249; 3 and 3′, strain A95210; 4 and 4′, strain 2955. The positions of PBPs 1a and 2b of strain R6 are indicated.

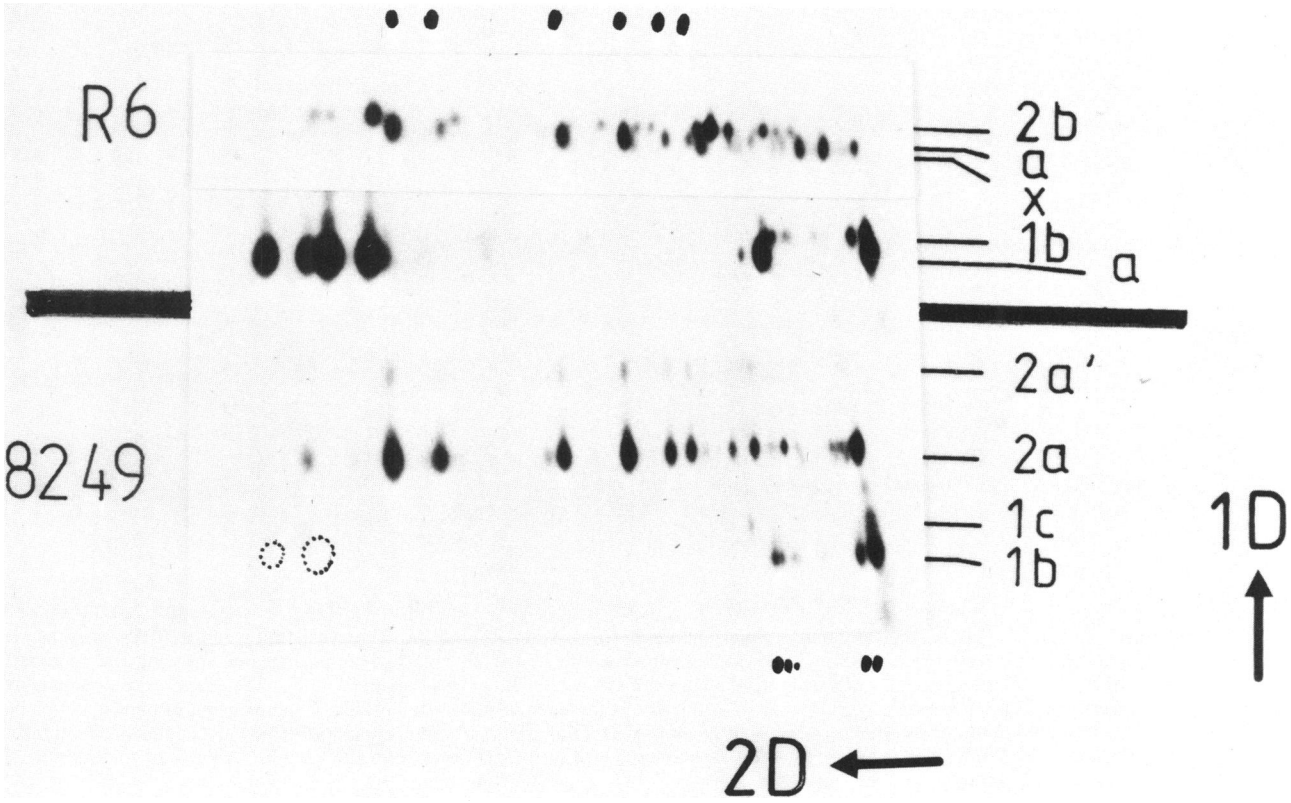

FIG. 3. Penicilloyl peptides of the high-molecular-weight PBPs of strains R6 and 8249 transformant pen 6. PBPs in membranes of the strains used (150 μg of protein) were labeled with [^{3}H]PA and separated in dimension 1 (1D) by 7.5% SDS-PAGE, followed by a dimension 2 (2D) run in 15% SDS-PAGE; the latter had V8 protease in the stacking gel (see the text for details). A fluorogram was prepared showing the radioactively labeled (beta-lactam-binding-site-containing) peptides. In the fluorogram of strain R6 membranes (upper part of figure), PBPs 1a and 1b ran quite close to each other in dimension 1 of the gels; this was also true for PBPs 2a, 2b, and 2x. To facilitate the identification of the peptides belonging to these PBPs, ink dot markers indicate the positions of some of the characteristic peptides (6) generated from PBP 2a (ink dots in the top margin) and PBP 1b (ink dots at the bottom). Faint spots are highlighted by dotted circles. In the fluorogram of strain 8249, no peptides corresponding to PBP 2b are visible adjacent to the peptides generated from PBP 2a. PBP 2a' is characteristic of strain 8249. The exposure time of the film was 1 day.

tions of the denatured proteins (eluted from SDS gels) of PBP 1a (Ellerbrok, Ph.D. dissertation), PBP 2b (Briese, Ph.D. dissertation), and PBP 3 (9) were used as antigens. The immunization methods were as follows. (i) PBP 1a. About 20 μg of protein was injected subcutaneously with complete Freund adjuvant into adult male rabbits, followed by three booster injections (10, 20, and 10 μg of protein, each with incomplete Freund adjuvant) at weeks 3, 5, and 9 after immunization. Serum titers were determined after week 11. (ii) PBP 2b. Two-month-old BALB/c mice were injected subcutaneously with 2 to 5 μg of protein in complete Freund adjuvant, followed by two booster injections (2 to 5 μg of protein, incomplete Freund adjuvant) 5 weeks apart. Serum was taken after 6 months. (iii) PBP 3. Rabbits received subcutaneous injections of 50 μg of protein (with complete Freund adjuvant) followed by two boosters (50 μg of protein, incomplete adjuvant) at weeks 4 and 6. Serum was taken after 5 months.

Blotting procedure. Proteins were transferred from SDS-PAGE onto nitrocellulose membranes (BA85, 0.45 μm pore size; Schleicher & Schuell GmbH, Frankfurt, Federal Republic of Germany) (21) with Tris-glycine buffer (20/200 mM; pH 8.3)–20% methanol in a TE42 Transphor electrophoresis unit (Hoefer Instruments, San Francisco, Calif.) for 2 h at 6 V/cm. The nitrocellulose membranes were sequentially incubated at room temperature in (i) phosphate-buffered saline–0.05% Tween (PBS-Tween) overnight, (ii) antiserum diluted 1:2,000 to 1:10,000 for 2 h in PBS-Tween, (iii) alkaline phosphatase-conjugated anti-rabbit or anti-mouse immunoglobulin G (Sigma Chemical Co., St. Louis, Mo.) in PBS-Tween. Between each stage, the membrane sheet was washed with PBS-Tween three and four times, respectively. Bound enzyme activity was revealed by staining with Nitro Blue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate by the method of Blake et al. (1), with the exception of the use of 0.1 M diethanolamine hydrochloride buffer (pH 9.8).

RESULTS

The PBP profiles of susceptible laboratory strain R6 and three highly penicillin-resistant clinical isolates of pneumococci, strains 8249, A95210, and 2955, are shown in Fig. 1. A common feature of the PBP patterns of the resistant bacteria was the absence of PBP 1a. Instead, each of the resistant pneumococci had high-molecular-size PBPs that are characteristic of the particular strain. The approximate molecular sizes of these PBPs are 96 kDa (in strain A95210), 94 kDa (in strain 2955), and 92 kDa (in strain 8249). Antiserum prepared against purified PBP 1a (Ellerbrok, Ph.D. dissertation) was used to test the possible relatedness of these high-molecular-size PBPs to one another.

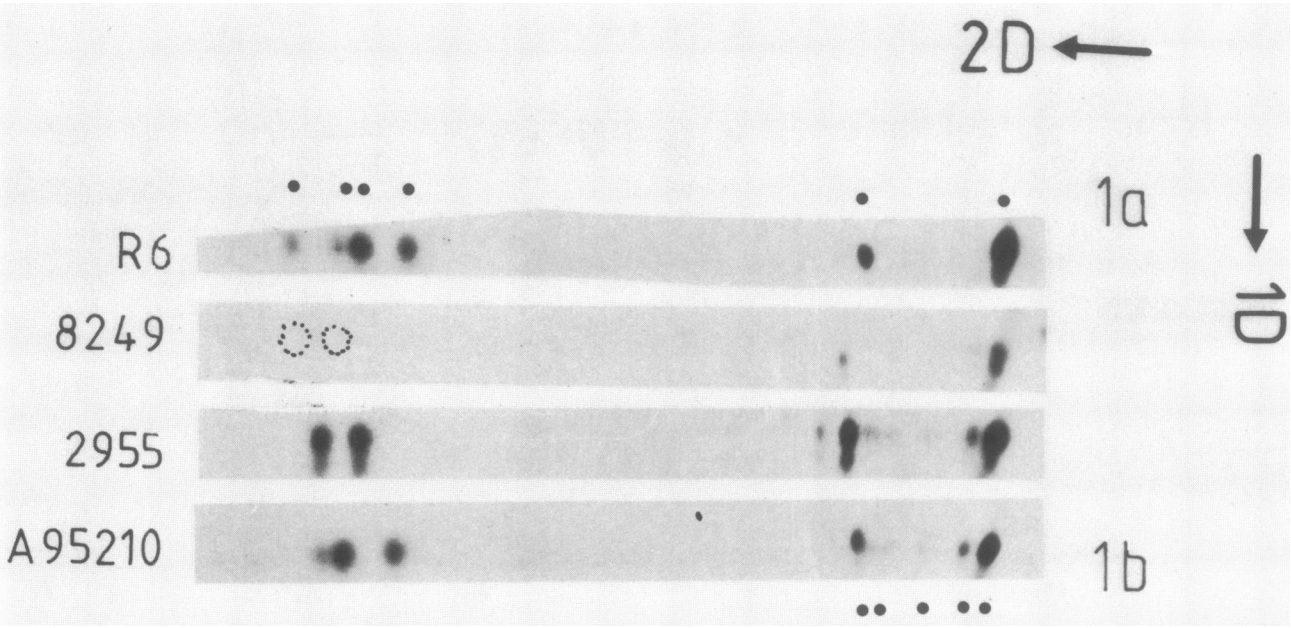

FIG. 4. Penicilloyl peptides of the highest-molecular-weight proteins from strain R6 and the three resistant strains. PBPs were labeled, separated, and digested during a dimension 2 SDS-PAGE as described in the legend to Fig. 3. The fluorograms showing the radioactive peptides derived from PBP of a molecular size higher than 85 kDa were cut out of the gel and aligned. Above the R6 gel, the dots mark the positions of characteristic peptides generated from PBP 1a of strain R6; at the bottom right, the dots mark the position of peptides typical of PBP 1b of strain R6. In strain 8249 (and its transformant, pen 6), only very faint spots, which correspond to PBP 1a peptides characteristic of strain R6 (open circles), could be visualized even after prolonged exposure of the gel because of the very low affinity of the beta-lactam. 1D and 2D, Dimension 1 and 2 SDS-PAGE, respectively.

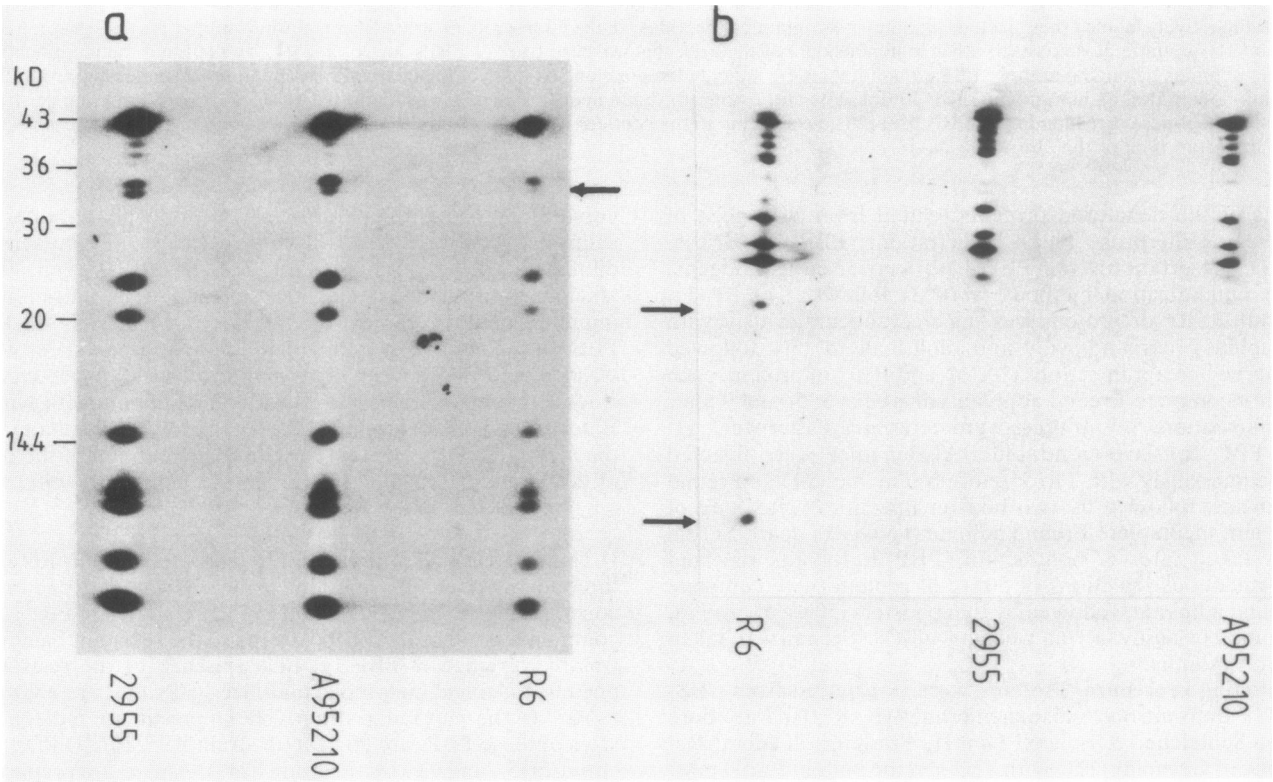

FIG. 5. Comparison of proteolytic peptides of PBP 3 from strains R6, 2955, and A95210. A two-dimensional gel for the determination of V8 protease peptides was prepared for PBP 3 of the three pneumococcal strains. Samples were prepared in duplicate. Panel a, Fluorograms of the [^{3}H]PA-labeled peptides. The arrow on the right points to a possible minor difference in the strain 2955-derived digest. Panel b,

Another peculiarity of strain 8249 reported earlier is that these bacteria do not seem to contain PBP 2b, one of the major PBPs of penicillin-susceptible pneumococci (27; Fig. 1). Of the pneumococcal PBPs, PBP 2b has the lowest relative affinity for benzylpenicillin even in susceptible cells (22), and it was shown that in strains of intermediate and high resistance this PBP undergoes a further stepwise decrease in antibiotic affinity (11, 27). The lack of detectable PBP 2b in the fluorograms of highly resistant strain 8249 may be the result of the poor binding capacity under the conditions of the penicillin binding assay. The availability of antibody against PBP 2b (Briese, Ph.D. dissertation; Ellerbrok, Ph.D. dissertation) allowed us to test this possibility also.

The results of immunoblots using rabbit anti-1a and mouse anti-2b antisera are shown in Fig. 2. It is quite clear that the antibodies against PBP 1a reacted with a single high-molecular-weight protein band not only in the sensitive membranes but in each one of the resistant membrane preparations as well and that the position of the bands corresponded precisely with the positions of the highest-molecular-size PBP on the fluorograms of the corresponding membrane preparations. Anti-2b antibodies reacted in all four samples with one band at a position corresponding to that of PBP 2b in the fluorogram of the susceptible bacteria. With both antisera, the reaction was strongest with the wild-type PBPs. This test did not allow us to distinguish between the two possibilities that either less PBP is present in the resistant strains (it should be noted that an equal amount of membrane protein per sample was used) or the same quantity of altered (i.e., immunologically less reactive) PBP is present.

No antiserum against PBP 2a was available. All resistant strains contain a PBP with the same M_r as that of PBP 2a. The identity of this protein was tested by using a two-dimensional gel system including partial proteolysis by V8 protease. With this method, each of the pneumococcal PBPs was shown to generate a characteristic peptide pattern (5, 6). A comparison of penicilloyl peptides derived from high-molecular-weight PBP of the susceptible wild-type pneumococci and those of strain 8249 is shown in Fig. 3. It is evident that the two PBPs that can be labeled with [^{3}H]PA in strain 8249 correspond to PBPs 1b and 2a. These proteins were also detected in the other two strains (results not shown).

The same technique revealed major differences between PBP 1a of the susceptible cells and the immunologically related high M_r PBPs of the resistant strains (Fig. 4). In the PBP 1a of susceptible cells, V8 protease digestion generated four characteristic peptides containing the beta-lactam binding site of molecular size ranging from 10 to 12.7 kDa (see ink dots on top left margin of Fig. 4). In all three resistant strains, this low M_r peptide pattern of PBP 1a was changed on the fluorogram, indicating that alterations must have occurred in the vicinity of the active center of these proteins.

Strain 2955 contains no PBP at the position corresponding to PBP 3 of the susceptible strain and the other resistant isolates (Fig. 1). Instead, there is a binding protein of higher molecular size (about 52 kDa instead of the 45-kDa PBP 3). To clarify the relationship of this protein to PBP 3, the SDS gels were analyzed by antibody prepared against PBP 3 of the susceptible pneumococci.

The 52-kDa PBP gave a strong positive reaction with antibodies prepared against PBP 3 from the susceptible strain, as did PBP 3 in the other resistant strains (results not documented). To determine whether the additional polypeptide material responsible for the increased size of PBP 3 in strain 2955 was located near the beta-lactam binding site, the patterns of [^{3}H]PA-labeled peptides, as well as the patterns of peptides which were recognized by anti-PBP 3 antiserum, were compared. Strain A95210 (and 8249; results not shown) was included to see whether some changes in the PBP 3 components of these strains could also be detected. The fluorogram of V8 protease-derived radioactive peptides of strain R6 and resistant strains 2955 and A95210 is shown in Fig. 5a. Only minor differences (see arrow) were revealed in strain 2955, and the great majority of the peptides appeared identical in all three strains. However, when a similar experiment was performed but the peptides of PBP 3 were detected with anti-PBP 3 antibodies (instead of radioactivity), several changes became apparent (Fig. 5b). Naturally, the peptides visible on the immunoblot need not be the same as those identified by fluorography; in fact, only very few of the peptides were found to be radioactive and antibody reacting as well. A clear shift toward higher molecular size occurred in all the peptides of PBP 3 from strain 2955 that were bigger than 20 kDa (Fig. 5b).

A small antigenic peptide of approximately 10 kDa detectable on the immunoblot of the PBP 3 of the wild-type susceptible cells appeared to be absent in the three resistant strains tested.

DISCUSSION

The application of immunological techniques and the comparison of peptide patterns generated by partial proteolysis allowed clarification of several of the complex differences between the PBPs of penicillin-susceptible and -resistant pneumococci.

The observations with the antibody against PBP 1a confirm the previous finding obtained with the penicillin binding technique concerning the absence of PBP 1a from resistant cells (20, 27). In addition, the strong positive immunological reaction elicited by the anti-PBP 1a antiserum with PBP 1c of strain 8249 and with the 94- and 92-kDa binding proteins of the other two resistant strains indicates that the highest-molecular-size binding proteins of these pneumococcal strains are antigenically related to PBP 1a. Previous studies with genetic transformants showed that the appearance of PBP 1c and the simultaneous disappearance of PBP 1a occur at a distinct level of penicillin resistance and that this change in the PBP pattern is preceded by a gradual decrease in the penicillin affinity of PBP 1a in transformants with lower and intermediate levels of resistance (27). It has been proposed that this process involves the gradual accumulation of point mutations within the gene for PBP 1a, lowering the capacity of the protein for antibiotic binding. At some point, this process may result in the generation of a processing signal, causing the proteolytic splitting of PBP 1a to 1c (12, 20). The immunological relatedness of PBPs 1a and 1c demonstrated in this report is consistent with this proposal. The analysis of peptide patterns generated from the highest-molecular-size PBPs indicates that some of the mutational alteration(s) must have occurred within the 10- to 12.7-kDa peptides containing

Immunoblot of the peptides, stained with anti-PBP3 antiserum produced against PBP 3 of the penicillin-susceptible (R6) wild strain. The arrows on the left show two peptides that are strongly reactive only in strain R6. Panels a and b are aligned so that the molecular sizes shown on the left are valid for both the fluorogram and the immunoblot.

the beta-lactam site, the enzymic active center. It is interesting that the changes in peptide pattern were different in the three resistant strains analyzed, suggesting that alternative mutational pathways exist for the remodeling of this PBP.

The immunological assay of the membranes of penicillin-resistant strain 8249 indicates that these bacteria continue to produce PBP 2b but that the greatly decreased penicillin affinity of this protein (12) no longer allows its detection by the [^{3}H]penicillin binding assay. PBP 2b is one of the physiologically important binding proteins of pneumococci (27). It might be expected, therefore, that the acquisition of low affinity for benzylpenicillin in PBP 2b of strain 8249 does not significantly alter its physiological function(s) in the resistant bacteria.

The immunological observations indicate that the 52-kDa binding protein of strain 2955 is closely related to PBP 3 of the susceptible pneumococci, a protein with DD-carboxypeptidase activity (9). Genetic transformation using DNA from strain 2955 indicates that the altered PBP 3 is not cotransformed during the introduction of penicillin resistance (A. Tomasz, unpublished observation).

The precise nature of structural differences between the larger-molecular-size (52-kDa) PBP 3 of strain 2955 and PBP 3 of the penicillin-susceptible reference strain is not known. Nevertheless, none of the unique, large (>20-kDa) peptides generated from the 52-kDa PBP by V8 protease and detected by the antiserum against PBP 3 contained the beta-lactam binding site. This suggests that the extra polypeptide material responsible for the larger molecular size of this protein lies within a 25-kDa piece, distant from the beta-lactam binding site.

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