ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 1980, p. 364-371
0066-4804/80/03-0364/08$02.00/0

Vol. 17, No. 3

Multiple Changes of Penicillin-Binding Proteins in Penicillin-Resistant Clinical Isolates of *Streptococcus pneumoniae*

REGINE HAKENBECK,† MARTHA TARPAY,[1] AND ALEXANDER TOMASZ[2]*

The Rockefeller University, New York, New York 10021,[2] and University of Oklahoma Health Sciences Center, Department of Pediatrics, Oklahoma City, Oklahoma 73190[1]

Penicillin-binding properties and characteristics of penicillin-binding proteins (PBPs) were investigated in several clinical isolates of *Streptococcus pneumoniae* differing in their susceptibilities to penicillin (minimal inhibitory concentration [MIC], 0.03 to 0.5 μg/ml) and compared with the penicillin-susceptible strain R36A (MIC, 0.07 μg/ml). Several changes accompanied the development of resistance: the relative affinity to penicillin of whole cells, isolated membranes, and two major PBPs after in vivo or in vitro labeling decreased (with increasing resistance). Furthermore, one additional PBP (2') appeared in four of five relatively resistant strains with an MIC of 0.25 μg/ml and higher. PBP 3 maintained the same high affinity toward penicillin in all strains under all labeling conditions.

Attempts to establish a correlation between penicillin susceptibility of bacteria (minimal inhibitory concentration [MIC] value) and the ability of these cells to bind the antibiotic have already been reported in the early literature (15). Eagle has documented such a relationship by comparing the penicillin-binding capacity of relatively resistant bacteria (*Escherichia coli*) with that of susceptible ones (e.g., *Streptococcus pneumoniae*; 4). On the other hand, he found little if any correlation when he examined a series of staphylococcal mutants differing in their degree of penicillin resistance (5). A more elaborate study by Edwards and Park using *Staphylococcus aureus* H revealed a correlation between the cellular binding of penicillins and cephalosporins and the corresponding growth-inhibitory concentrations (6). The major difficulty of these studies was the low specific activity of the radioactive penicillin used which prevented studies at MIC and below MIC levels.

In another, more recent approach, radioautography has been introduced to visualize individual penicillin-binding proteins (PBPs; 24). In all organisms evaluated so far, these proteins appear to be membrane-bound components of poorly understood biochemical function. As an approach to tentatively identifying the killing target for penicillin, Reynolds et al. (20) have looked for the PBP with the highest affinity toward the antibiotic after in vivo labeling. With their procedure, PBP 1 appeared as the most sensitive penicillin-binding component in *Bacillus megaterium* (20). An alternative method is to examine possible changes in PBPs in bacterial mutants differing in levels of penicillin resistance. It has been found that mecillinam-resistant *E. coli* contain a PBP 2 with decreased affinity toward that beta-lactam, and cloxacillin-resistant *Bacillus subtilis* PBP 2 exhibited similar behavior (3, 25). A tendency for lower penicillin binding has been described for membranes isolated from penicillin-resistant gonococci (21).

Recently, Giles and Reynolds have examined the PBPs of a cloxacillin-resistant *B. megaterium* and its antibiotic-susceptible revertants. Two changes appeared to accompany drug resistance: a decrease in affinity for PBP 1 and an increase in the amount of PBP 2 (8).

In this report, we describe penicillin-binding characteristics of clinical isolates of *S. pneumoniae* strains with different degrees of susceptibility to penicillin. The use of radioactive penicillin with a high specific activity has allowed rapid labeling of the PBPs of the bacteria both in vivo and in vitro. It was found that as the level of resistance increased, the penicillin-binding capacity of whole cells as well as isolated membranes declined. This change was reflected as an apparent decrease in the affinity of the PBP 1 and PBP 2 groups, but only relatively minor quantitative changes were observable in the PBP 3 of the penicillin-resistant bacteria. In addition, in strains of higher resistance level (MIC, 0.25 μg/ml and higher), PBP 2b was greatly diminished and a new protein of higher molecular weight (PBP 2') appeared. One strain of intermediate resistance showed still another alteration: the disappearance of PBP 1a and an increase in the intensity of a PBP with the mobility of PBP 1b.

† Present address: Max-Planck Institut fur Molekulare Genetik, D 1000 Berlin 33, West Germany.

MATERIALS AND METHODS

Bacterial strains and media. All encapsulated pneumococci were clinical isolates and were identified by typical colony morphology and alpha-hemolysis (Table 1). All strains showed uniform susceptibility to optochin (ethyl hydrocupreine hydrochloride) and remained bile soluble. By the chromogenic cephalosporin test (19), none of the strains showed beta-lactamase production. Antibiotic susceptibility testing was performed by the disk diffusion technique (1), using a 6-μg penicillin G disk on Mueller-Hinton agar with 5% sheep blood. MICs and minimal bactericidal concentrations were routinely determined by a broth dilution method (M. Tarpay, manuscript in preparation). Serotyping of the strains was performed by the Center for Disease Control. Independent susceptibility testing of all clinical isolates was also performed by the Center for Disease Control as part of a surveillance study for relatively resistant strains of *S. pneumoniae* in Oklahoma (23). The noncapsulated, penicillin-susceptible *S. pneumoniae* R36A (wild type, Rockefeller University laboratory stock) was used as a reference strain. *S. pneumoniae* cwl is a penicillin-tolerant, autolysin-defective transformant of strain R36A (27). Bacteria were grown at 37°C without aeration in C medium (26) supplemented with yeast extract (1% final concentration; Difco Laboratories). Growth was monitored with a Coleman nephelometer.

Membrane preparation. Cells of a 500-ml culture were harvested at a cell concentration of about 1.5×10^8 colony-forming units (CFU)/ml, washed once with ice-cold saline, and resuspended in 5 ml of 10 mM PO_4 buffer, pH 6.8. All further manipulations were carried out at 4°C. Cells were disrupted in a Mickle cell disintegrator (17), and whole cells and cell walls were removed by centrifugation at 27,000 × *g* for 15 min. Membranes were pelleted and washed twice at 45,000 rpm, using an SW50.1 rotor in a Beckman model L5-50 ultracentrifuge. They were stored at −70°C at a protein concentration between 30 and 45 mg/ml, determined by the method of Lowry et al. (14).

TABLE 1. *Strains of S. pneumoniae used in this study*[a]

Strain	Type	Benzylpenicillin MIC	
		μg/ml	nmol/ml
R36A (wild type)	Nonencapsulated	0.007	0.02
cwl (penicillin tolerant)	Nonencapsulated	0.007	0.02
521	6	0.03	0.09
690	19	0.03	0.09
532	14	0.03	0.09
537	14	0.1	0.3
662	6	0.25	0.74
R	14	0.25	0.74
B	14	0.25	0.74
M	14	0.5	1.5
Mo	23	0.5	1.5

[a] For details of typing and MIC determination, see Materials and Methods.

Binding of [^{3}H]penicillin to whole cells. Exponentially growing cells were harvested at a cell concentration of 1.5×10^8 CFU/ml, centrifuged, and suspended in one-eighth the initial volume of buffer. Samples of 200 μl were added to the various dilutions of [^{3}H]penicillin and incubated for 10 min at 30°C. Binding was stopped with nonradioactive benzylpenicillin (300 μg in 5 μl), and samples were put in an ice bath. Cells were immediately collected on a membrane filter, (pore size, 0.45 μm; Millipore Corp.), which was saturated with nonradioactive benzylpenicillin, and washed with ethanol. Filters were dried and counted in 5 ml of toluene-based scintillation fluid.

Binding of [^{3}H]penicillin to membranes. Incubation of membranes with [^{3}H]penicillin was carried out in a total volume of 60 μl containing 240 μg of membrane protein. Binding was allowed to take place at 30°C for 10 min; then an excess of nonradioactive penicillin and 2 ml of ice-cold trichloroacetic acid were added. Samples were collected and washed with trichloroacetic acid on membrane filters (0.45 μm) that had been soaked in a nonradioactive penicillin solution. After drying of the filters, radioactivity was determined as above.

Labeling of PBPs with [^{3}H]penicillin. To identify pneumococcal PBPs, three labeling regimens were applied.

(i) In vivo labeling. Samples of 1 ml of exponentially growing cells at a cell concentration of 5×10^7 CFU/ml were incubated with the various dilutions of penicillin at 37°C for 10 min. The binding was stopped by the addition of 5 μl of nonradioactive benzylpenicillin (approximately 300 μg); then cells were pelleted for 30 s at 12,000 × *g*, suspended in 50 μl of 10 mM PO_4 buffer (pH 6.8), and lysed within 5 min upon the addition of 2.5 μl of 20% Sarkosyl NL-97. A 25-μl amount of sample dilution buffer and 10 μl of mercaptoethanol were added, and samples were boiled for 2 min and applied to a polyacrylamide slab gel.

(ii) In vitro labeling of cell lysates. The same culture as for the in vivo labeling was used. At a concentration of 5×10^7 cells per ml, bacteria were centrifuged and resuspended in 1/20 of the initial volume, using 10 mM PO_4 buffer (pH 6.8) containing 1% deoxycholate. Cells were lysed within 5 min at 37°C, and 50-μl portions (containing the equivalent of 1 ml of culture) were incubated with [^{3}H]penicillin for 10 min at 37°C. The reaction was terminated, and samples were prepared for gel electrophoresis as described above.

(iii) Labeling of membranes. Membranes were incubated with various concentrations of [^{3}H]penicillin in a total volume of 50 μl containing 100 μg of membrane protein at 30°C for 10 min. The reaction was terminated, and samples were prepared for gel electrophoresis as described for in vivo labeling.

Gel electrophoresis and characterization of PBPs. Discontinuous sodium dodecyl sulfate-polyacrylamide slab gels were prepared and run as described by Laemmli and Favre (12), except that the concentrations of acrylamide and *N,N'*-methylenebisacrylamide were 7 and 0.117% in the separating gel

and 3 and 0.05% in the stacking gel. Electrophoresis was carried out at a constant voltage of 60 V until the tracking dye had just entered the separating gel and at 150 V thereafter. Gels were stained with Coomassie brilliant blue by the method of Fairbanks et al. (7) and destained with several changes of 30% methanol plus 10% acetic acid. Fluorography was done according to the method of Bonner and Laskey (2), and the gels were exposed to presensitized Kodak X-Omat XR-2 film (13). The exposure time for the fluorograms was 2 to 6 days at −70°C. The intensity of the bands of the fluorograms was determined with a Gelman type ACD-15 automatic computing densitometer.

Antibiotics and other reagents. Benzyl-[^{3}H]penicillin, ethylpiperidinium salt (31 Ci/mmol), was the generous gift of E. O. Stapley, Merck & Co., Inc., Rahway, N.J. The compound was stored in acetone at −20°C. Immediately before the experiments, the acetone was replaced by an equal volume of 10 mM PO_4 buffer (pH 6.8) and further diluted in the same buffer. Benzylpenicillin, potassium salt, was obtained from Eli Lilly & Co., Indianapolis, Ind. Chromogenic cephalosporin substrate was provided by C. O'Callaghan, Glaxo Research Ltd., Greenford, Middlesex, England. Acrylamide and *N,N'*-methylenebisacrylamide were electrophoresis grade (Bio-Rad, Rockville, N.Y.). All other chemicals were reagent-grade, commercially available products.

RESULTS

Binding of [^{3}H]penicillin to whole cells. A concentrated suspension of cells in buffer was used to determine the penicillin-binding capacity of the different strains. The amount of antibiotic bound at different concentrations to the cells is shown in Fig. 1. Although not totally proportional, a clear inverse relationship between susceptibility of the strains and their capacity to bind penicillin was seen. Binding was apparent at 0.007 μg of penicillin per ml (MIC for R36A and cwl) in the susceptible strains, whereas the binding to the highly resistant strains required concentrations of 0.25 to 1.5 μg/ml, corresponding roughly to their MICs.

Binding of penicillin to membranes. Figure 2 shows the amount of penicillin bound to crude membranes prepared from different strains (see Materials and Methods for details). Again, a marked reduction in the capacity to bind penicillin was observed in the membranes derived from the more resistant isolates. Results cannot be directly compared with the binding of whole cells in terms of absolute amounts because of the difference in protein concentrations used.

PBP pattern in different strains. In nonencapsulated pneumococci, five penicillin-binding components are present, designated 1a, 1b, 2a, 2b, and 3 (R. Williamson et al., FEMS Lett., in press). The high specific activity of the [^{3}H]penicillin makes it possible to label exponentially growing cells at the MIC and even below the MIC level.

Figure 3 shows a comparison of the PBPs after in vivo labeling between the nonencapsulated R36A strain and some of the clinical isolates. The patterns obtained after in vitro labeling were identical in each case. The most striking

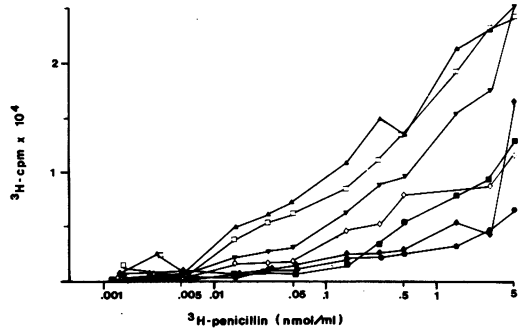

FIG. 1. *Binding of [^{3}H]penicillin to cells of S. pneumoniae. Exponentially growing cells were harvested at a cell concentration of 1.5 × 10^8 CFU/ml, centrifuged, and concentrated eightfold in buffer. Portions of 200 μl were added to the various dilutions of [^{3}H]penicillin and incubated for 10 min at 30°C. The binding was stopped with an excess of nonradioactive penicillin, samples were collected on a membrane filter, and radioactivity was determined. Symbols for the different strains and MICs (nanomoles per milliliter): (▲) cwl (0.02); (■) M (1.5); (▼) R36A (0.02); (◆) R (0.74); (□) 690 (0.09); (●) MO (1.5); (◇) 537 (0.3).*

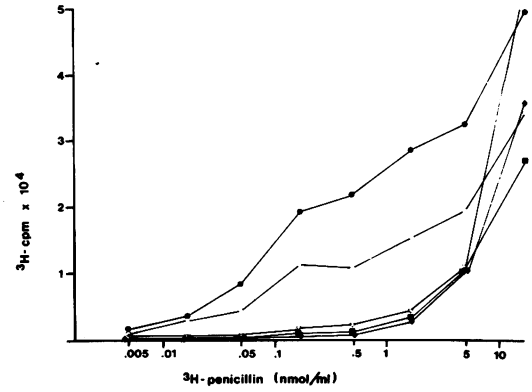

FIG. 2. *Binding of [^{3}H]penicillin to membranes isolated from various S. pneumoniae strains. Membranes were prepared as described in Materials and Methods. Binding of [^{3}H]penicillin was carried out in a total volume of 60 μl, using 240 μg of membrane protein. After incubation for 10 min at 30°C, an excess of nonradioactive penicillin (300 μg in 5 μl) and ice-cold trichloroacetic acid (2 ml of a 10% solution) were added, samples were collected and washed on a membrane filter, and radioactivity was determined. Symbols for the strains and MICs (nanomoles per milliliter): (●) R36A (0.02); (○) 521 (0.09); (▽) 537 (0.3); (■) B (0.74); (◆) 662 (0.74).*

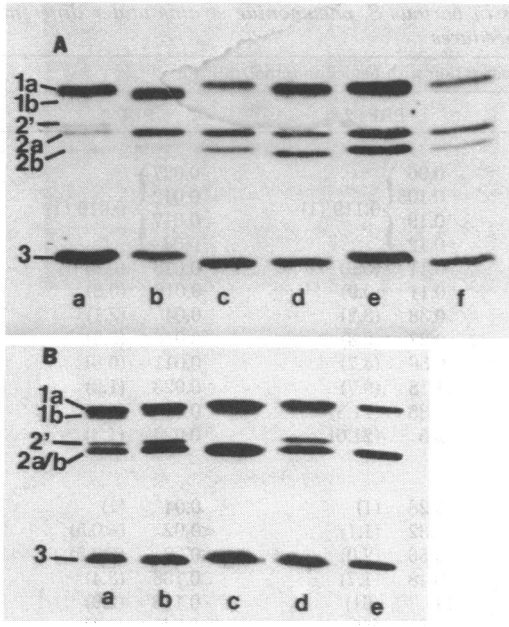

FIG. 3. *PBPs in the susceptible strain R36A and different resistant isolates. Exponentially growing bacteria (1 ml of cells at approximately 5 × 10^7 to 2 × 10^8 CFU/ml) were labeled with [^{3}H]penicillin (1 nmol/ml) for 10 min at 37°C. Labeling was terminated by the addition of nonradioactive penicillin. After lysis of the cells with Sarkosyl, proteins were fractionated by sodium dodecyl sulfate-polyacrylamide electrophoresis and PBPs were detected by fluorography (see Materials and Methods). Differences apparent in the total amounts of PBPs (e.g., slots e versus f in panel a) are due to the different amounts of proteins (corresponding to variations in cell concentrations). Strains and MICs (nanomoles per milliliter) are as follows. A: (a) R (0.74); (b) 537 (0.3); (c) R36A (0.02); (d) 532 (0.09); (e) 521 (0.09); (f) R36A (0.02). B: (a) B (0.74); (b) M (1.5); (c) 521 (0.09); (d) Mo (1.5); (e) R36A (0.02).*

feature was the appearance of one additional PBP (PBP 2') in the more resistant strains (strain R in Fig. 3A and strains B, M, and Mo in Fig. 3B). This phenomenon was observed in four of the five strains with a penicillin MIC of 0.25 μg/ml and higher and in none of the low-resistance pneumococci. One strain showed loss of PBP 1a combined with an increase in penicillin bound to a protein at the position of PBP 1b (strain 537; Fig. 3A). It should be noted that separation of PBP 2a and 2b depended on the lot of polyacrylamide, and this fact accounts for the somewhat different resolution of PBP group 2 in Fig. 3A versus 3B.

Correlation between MIC value of the strains and affinity to penicillin of the PBPs. To compare labeling in vivo and in vitro, the different strains were harvested carefully at the same cell density. In each set of experiments, strain R36A was included as a standard for calibrating the affinities. Autoradiograms of in vivo labeling with decreasing amounts of penicillin are represented in Fig. 4 for strains R36A and M. A distinct shift in saturation was seen in PBPs 1 and especially in PBPs 2 in the resistant strain. The novel PBP 2' in strain M appeared to have an even lower affinity than PBP 2a in this strain.

In Table 2, the 50% saturation values of the PBPs are summarized for the different strains with all three labeling regimens. PBPs 1a and 1b as well as 2a, 2b, and 2' were not completely separable from each other in the densitometer tracings and therefore were treated as a single PBP group. In the susceptible strain (R36A), both 1b and 2b had lower affinities toward penicillin than 1a and 2a; therefore, the 50% saturation values for PBPs 1 and 2 were higher than expected for 1a and 2a alone. With increasing resistance, a shift in the affinity for penicillin in

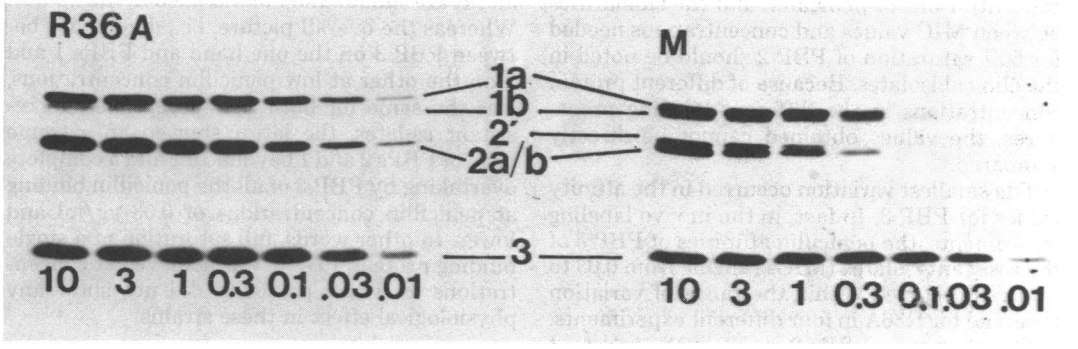

FIG. 4. *Sensitivity of PBPs to [^{3}H]penicillin in S. pneumoniae R36A and M after in vivo labeling. A 1-ml amount of exponentially growing cells was incubated for 10 min at 37°C with the various penicillin concentrations as indicated. Cells were lysed, proteins were separated on a sodium dodecyl sulfate-polyacrylamide slab gel, and fluorograms were developed.*

TABLE 2. *Fifty percent saturation values for the PBPs in various S. pneumoniae strains under different labeling procedures*

Labeling strain	Penicillin MIC (nmol/ml)	50% saturation with penicillin (nmol/ml)[a] for: PBPs 1		PBPs 2		PBP 3	
In vivo							
R36A	0.02	0.06, 0.043, 0.08, 0.034	0.054 (1)[b]	0.06, 0.105, 0.19, 0.12	0.119 (1)	0.027, 0.012, 0.017, 0.04	0.019 (1)
cwl	0.02	0.06	(1.1)	0.11	(0.9)	0.015	(0.8)
521	0.09	0.04	(0.7)	0.11	(0.9)	0.016	(0.8)
532	0.09	0.095	(1.7)	0.38	(3.2)	0.04	(2.1)
537	0.3	0.036	(0.7)	0.27	(2.3)	0.016	(0.8)
R	0.74	0.23	(4.3)	0.56	(4.7)	0.011	(0.6)
B	0.74	0.49	(9.1)	1.15	(9.7)	0.023	(1.2)
M	1.5	0.39	(7.2)	1.35	(11.3)	0.017	(0.9)
Mo	1.5	1.15	(21.3)	2.5	(21.0)	0.024	(1.3)
In vitro (cell lysates)							
R36A	0.02	0.1	(1)	0.28	(1)	0.04	(1)
521	0.09	0.112	(1.1)	0.32	(1.1)	<0.02	(<0.5)
532	0.09	0.36	(3.6)	0.56	(2.0)	<0.02	(<0.5)
537	0.3	0.12	(1.2)	0.48	(1.7)	0.136	(3.4)
B	0.74	0.52	(5.2)	17	(61)	0.116	(2.9)
M	1.5	0.58	(5.8)	12.8	(46)	<0.04	(<1)
Mo	1.5	0.1	(10)	16	(57)	0.04	(1)
In vitro (membranes)							
R36A	0.02	0.014	(1)	0.024	(1)	0.0074	(1)
521	0.09	0.032	(2.3)	0.04	(1.7)	0.0036	(0.5)
537	0.3	0.104	(7.4)	0.4	(17)	0.034	(4.7)
662	0.74	0.05	(3.6)	0.56	(23.3)	0.05	(6.7)
B	0.74	0.3	(21.4)	1.2	(50)	0.074	(10)

[a] All values are given in nanomoles per milliliter because of the different molecular weight for the radioactive benzylpenicillin when compared with the cold potassium benzylpenicillin.

[b] Numbers in parentheses indicate the relative shift in saturation compared with the susceptible strain R36A (nanomoles needed to obtain 50% saturation in the resistant strain divided by nanomoles needed to obtain 50% saturation in the susceptible R36A strain).

PBPs 1 and 2 became more and more apparent. The differences were clearly present in the "real" in vivo situation using exponentially growing bacteria and physiologically active (MIC) concentrations of penicillin, and the similarities between MIC values and concentrations needed for 50% saturation of PBP 2 should be noted in the clinical isolates. Because of different protein concentrations in the different labeling procedures, the values obtained cannot be directly compared.

The smallest variation occurred in the affinity values for PBP 3. In fact, in the in vivo labeling experiments, the penicillin affinities of PBP 3 of the resistant isolates (MICs ranging from 0.03 to 0.5 μg/ml) stayed within the range of variation observed for R36A in four different experiments.

The change in PBPs 2 and 1 in the resistant strains can be documented in another way by plotting the proportion of radioactive penicillin bound to the individual PBPs at different antibiotic concentrations. In Fig. 5, these values are shown for strains M and Mo in comparison with the penicillin-susceptible reference strain (R36A) after in vivo labeling (data for strains B and R are similar and not included in the figure). Whereas the overall picture, i.e., divergence between PBP 3 on the one hand and PBPs 1 and 2 on the other at low penicillin concentrations, was the same for both the susceptible and resistant isolates, the latter showed an extreme drop of PBPs 2 and 1 beyond 10% and a complete overtaking by PBP 3 of all the penicillin binding at penicillin concentrations of 0.03 μg/ml and lower. In other words, full saturation of a single binding protein, PBP 3, was achieved at concentrations at which penicillin did not show any physiological effect in these strains.

DISCUSSION

The isolation of pneumococci with moderate and relatively high levels of penicillin resistance

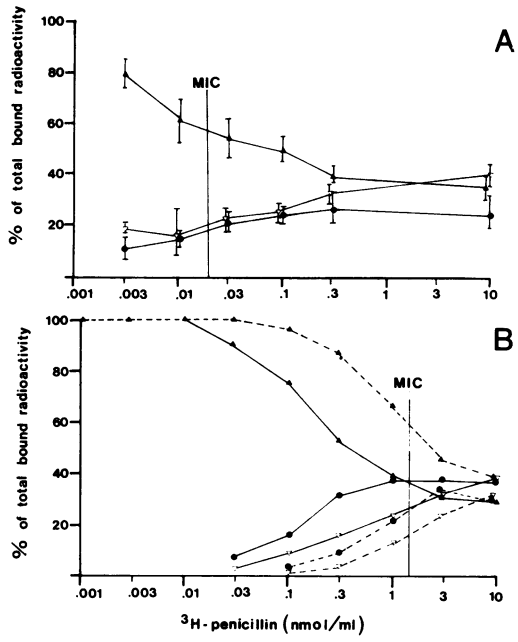

FIG. 5. *Percentage of total radioactive penicillin bound to individual PBPs at various antibiotic concentrations after in vivo labeling. Values were obtained after scanning fluorograms with a Gelman densitometer. Bars in (A) indicate extreme values obtained from four independent experiments. Exposure time was 2 to 5 days in (A) and 5 days in (B). (A) R36A; (B) M* (----) *and Mo* (——). *Symbols for PBPs:* (●) *PBP 1 group;* (▽) *PBP 2 group;* (▲) *PBP 3.*

has been reported in a number of clinical surveys (10, 11, 16, 18, 23). The studies described here were initiated in an attempt to understand the mechanism of resistance in a set of pneumococcal strains isolated from children at the Oklahoma Childrens Memorial Hospital. The nine strains represent four different capsular types, and the MIC values for benzylpenicillin range from 0.03 to 0.5 μg/ml. The MIC value for the penicillin-susceptible strain R36A is 0.007 μg/ml.

Our main approach to this problem has been to use radioactive penicillin to determine cellular antibiotic binding and to characterize PBPs in the penicillin-resistant pneumococci. PBPs are presumably penicillin-sensitive enzymes of cell wall metabolism, and change in the reactivity of these proteins to the antibiotic may contribute to cellular penicillin resistance.

We observed three types of biochemical changes in the penicillin-resistant pneumococci: (i) decrease in the cellular capacity for penicillin binding; (ii) decrease in the capacity of isolated membranes for penicillin binding; and (iii) a number of distinct changes in both the antibiotic affinities and the biochemical nature of PBPs. These latter changes were observed during both in vivo and in vitro labeling of the PBPs.

These findings allow several conclusions bearing on the mechanism of penicillin resistance. The decline in cellular penicillin-binding capacity closely parallels the decline in penicillin susceptibility of the bacteria. However, a change in the permeability properties of the cell wall does not seem to be a major factor in this phenomenon, since membranes prepared from the resistant bacteria also exhibit the decrease in binding capacity in proportion with the increase in the strains' MIC values. Furthermore, the decreased penicillin binding of cells and membranes can be accounted for in terms of the lowered affinity of two groups of PBPs—PBPs 1 and 2—which together are responsible for a maximum of about 60 to 70% of cellular penicillin binding (measured at high concentrations of the antibiotic). The PBP affinity changes can be observed during exposure of both live cells (in vivo labeling) and membrane preparations (in vitro labeling) to the radioactive antibiotic. This observation suggests that selective changes in cell wall permeability to penicillin do not contribute to the PBP alterations observed.

The changes in PBPs in the resistant bacteria involve a decrease in the affinity of PBP groups 2 and 1 for penicillin. An additional change, reduction in the quantity of PBP 2b and the appearance of a novel PBP (2′) of a lower electrophoretic mobility, may be observed in strains of higher resistance levels (MIC ≧ 0.25 μg/ml). If one arranges the resistant strains in the order of increasing MIC values, one has the impression that the alterations in PBPs represent gradual and cumulative changes: the decreased penicillin affinities of PBP 2 evident already in the low-resistance strains (MIC four times higher than in susceptible strains) are combined with a gradual decrease in the drug affinity of PBP 1; and the additional change indicated by the fading of PBP 2b (and the appearance of PBP 2′) is observed in the high-resistance strains.

The pneumococcal strains compared here for their PBP patterns are independent clinical isolates of unknown genetic background. Nevertheless, the gradual and multiple changes in PBPs are not simply casual features of the individual strains, since the same types of PBP alterations have been observed in penicillin-resistant genetic transformants constructed by crossing deoxyribonucleic acid from a highly penicillin-resistant South African strain of pneumococcus into penicillin-susceptible R36A cells (28). Whether the appearance of PBP 2′ represents a

new class of protein or some modification of PBP 2b remains to be determined. We are in the process of constructing penicillin-resistant isogenic strains by genetic transformation with donor deoxyribonucleic acids isolated from the clinical strains.

Only minor changes (if any) were observed in PBP 3 in terms of molecular weight and affinity for penicillin during development of resistance. It should be noted that PBP 3 has the highest affinity for penicillin of all strains examined and under all labeling conditions. The lack of any apparent change in this PBP in bacteria that differ vastly in their penicillin resistance is in contrast with the observations made in Reynolds' laboratory with *B. megaterium*. In that bacterium, it was the physiologically most cloxacillin-sensitive PBP 1 that was shown to fluctuate in its antibiotic affinity in cloxacillin-resistant mutants and their antibiotic-susceptible revertants (8). Determination of the physiological role of the pneumococcal PBP 3 will require different experimental approaches.

The complex alterations in PBPs that accompany the acquisition of penicillin resistance in pneumococci indicate that, at least in terms of the number of the affected PBPs, pneumococci contain not one but a number of "killing targets." A more realistic appraisal of the contribution of the observed PBP alterations to penicillin resistance will require the identification of the biochemical functions of the pneumococcal PBPs.

Changes in PBPs have been observed recently in membranes prepared from penicillin-resistant strains of *S. pneumoniae* (P. B. Percheson, and L. E. Bryan, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 19th, Boston, Mass., abstr. no. 488, 1979).

ACKNOWLEDGMENTS

This investigation was supported by a fellowship from the Deutsche Forschungsgemeinschaft (to R.H.), by grants from the National Institute of Health (Public Health Service grant AI 16170) and the National Science Foundation (PCM 7812770), and by a small grant award of the University of Oklahoma Health Sciences Center (to M.T.).

LITERATURE CITED

1. **Barry, A. L., and L. D. Sabath.** Special tests: bactericidal activity and activity of antimicrobics in combination, p. 431–435. *In* E. H. Lennette, E. H. Spaulding, and J. P. Truant (ed.), Manual of clinical microbiology, 2nd ed. American Society for Microbiology, Washington, D.C.
2. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83–88.
3. **Buchanan, C. E., and J. L. Strominger.** 1976. Altered penicillin binding components in penicillin-resistant mutants of *Bacillus subtilis*. Proc. Natl. Acad. Sci. U.S.A. **73**:1816–1820.
4. **Eagle, H.** 1954. The binding of penicillin in relation to its cytotoxic action. I. Correlation between the penicillin sensitivity and combining activity of intact bacteria and cell free extracts. J. Exp. Med. **99**:207–226.
5. **Eagle, H.** 1954. The binding of penicillin in relation to its cytotoxic action. II. The reactivity with penicillin of resistant variants of Streptococci, Pneumococci, and Staphylococci. J. Exp. Med. **100**:103–115.
6. **Edwards, J. R., and J. T. Park.** 1969. Correlation between growth inhibition and the binding of various penicillins and cephalosporins to *Staphylococcus aureus*. J. Bacteriol. **99**:459–462.
7. **Fairbanks, G., T. L. Steck, and D. H. F. Wallach.** 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry **10**: 2606–2717.
8. **Giles, A. F., and P. E. Reynolds.** 1979. *Bacillus megaterium* resistance to cloxacillin accompanied by a compensatory change in penicillin binding proteins. Nature (London) **280**:167–168.
9. **Guymon, L. F., D. C. Walstad, and P. F. Sparling.** 1978. Cell envelope alterations in antibiotic-sensitive and -resistant strains of *Neisseria gonorrhoeae*. J. Bacteriol. **136**:391–401.
10. **Hansman, D., H. Glasgow, J. Sturt, L. Devitt, and R. Douglas.** 1971. Increased resistance to penicillin of pneumococci isolated from man. N. Engl. J. Med. **284**: 175–177.
11. **Jacobs, M. R., H. J. Koornhof, R. M. Robins-Browne, C. M. Stevenson, Z. A. Vermaak, I. Freiman, G. B. Miller, M. A. Witcomb, M. Isaacson, J. I. Ward, and R. Austrian.** 1978. Emergence of multiply resistant pneumococci. N. Engl. J. Med. **299**:735–740.
12. **Laemmli, U. K., and M. Favre.** 1973. Maturation of the head of bacteriophage T4. I. DNA packing events. J. Mol. Biol. **80**:575–599.
13. **Laskey, R. A., and A. D. Mills.** 1975. Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. Eur. J. Biochem. **56**:335–341.
14. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
15. **Maass, E. A., and M. J. Johnson.** 1949. Penicillin uptake by bacterial cells. J. Bacteriol. **57**:415–422.
16. **Mace, J. W., S. Janik, and R. C. Sauer.** 1977. Penicillin resistant pneumococcal meningitis in an immunocompromised infant. J. Pediatr. **91**:506–507.
17. **Mosser, J. L., and A. Tomasz.** 1970. Choline-containing teichoic acid as a structural component of pneumococcal cell wall and its role in sensitivity to lysis by an autolytic enzyme. J. Biol. Chem. **245**:287–298.
18. **Naragi, S., G. P. Kirkpatrick, and S. Kabins.** 1974. Relapsing pneumococcal meningitis: isolation of an organism with decreased susceptibility to penicillin G. J. Pediatr. **85**:671–673.
19. **O'Callaghan, C. H., A. Morris, S. M. Kirby, and A. H. Shingler.** 1972. Novel method for detection of β-lactamases by using a chromogenic cephalosporin substrate. Antimicrob. Agents Chemother. **1**:283–288.
20. **Reynolds, P. E., S. T. Shepherd, and H. A. Chase.** 1978. Identification of the binding protein which may be the target of penicillin action in *Bacillus megaterium*. Nature (London) **271**:568–570.
21. **Rodriguez, W. J., and A. K. Saz.** 1978. Differential binding of penicillin by membrane fractions from penicillin-susceptible and -resistant gonococci. Antimicrob. Agents Chemother. **13**:589–597.
22. **Rowley, D., P. D. Cooper, P. W. Roberts, and E. L. Smith.** 1950. The site of action of penicillin. I. Uptake of penicillin on bacteria. Biochem. J. **46**:157–161.
23. **Saah, A. J., J. P. Mallonee, M. Tarpay, C. P. Thornsberry, M. A. Roberts, and E. R. Rhoades.** 1980.

Relative resistance to penicillin in the pneumococcus. A prevalence and case control study. J. Am. Med. Assoc., in press.

24. **Spratt, B. G.** 1977. Properties of the penicillin binding proteins of *Escherichia coli* K 12. Eur. J. Biochem. **72:** 341-352.
25. **Spratt, B. G.** 1978. *Escherichia coli* resistance to β-lactam antibiotics through a decrease in the affinity of a target for lethality. Nature (London) **274:**713-715.
26. **Tomasz, A.** 1970. Cellular metabolism in genetic transformation of pneumococci: requirement for protein synthesis during induction of competence. J. Bacteriol. **101:**860-871.
27. **Waks, S., and A. Tomasz.** 1978. Secretion of cell wall polymers into the growth medium of lysis defective pneumococci during treatment with penicillin and other inhibitors of cell wall synthesis. Antimicrob. Agents Chemother. **13:**293-301.
28. **Zighelboim, S., and A. Tomasz.** 1980. Penicillin-binding proteins of mutiply antibiotic resistant South African strains of *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **17:** 434-442.