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Possible Mechanism of Decreased Susceptibility of *Neisseria gonorrhoeae* to Penicillin

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By use of ^{14}C-labeled benzyl penicillin, it has been established that β-lactamases and/or acylases play no role in the resistance of *Neisseria gonorrhoeae* to penicillin. It has been found, however, that very susceptible strains of the organisms (minimal inhibitory concentration, 0.008 μg/ml) bind 10 to 15 times as much penicillin as do moderately to highly resistant strains of the gonoccoccus (minimal inhibitory concentration, 0.125 to 2.0 μg/ml). It is postulated that this degree of change in binding components of the whole cell and whole cytoplasmic membrane is sufficient to account for the decreased susceptibility of the organism to penicillin.

Over the last 20 years, there have been numerous reports indicating a significant decrease in susceptibility of *Neisseria gonorrhoeae* to benzyl and certain other penicillins. In 1970, Master (17) surveyed the susceptibility of over 2,000 gonococcal strains isolated in the United States over the previous 14 years. Between 1950 and 1955, none of the 771 strains tested was resistant to concentrations greater than 0.3 μg/ml. In contrast, in 1965, 5% of the strains observed required higher antibiotic concentrations for inhibition. By 1969, of 649 isolates, 14% required greater than 0.5 μg/ml for inhibition. This development has had profound effects on both the epidemiology and therapy of gonorrhoea. The treatment of acute urethritis in males and chronic or cryptic infections in females has been compromised. Not only is decreased susceptibility to penicillin encountered, but also some strains have been found which are multiply resistant to penicillin, streptomycin, spiromycin, chloramphenicol, and tetracycline (16). This latter characteristic, though not yet documented, may conceivably be related to the demonstration of pili in virulent strains.

We have secured from the Center for Disease Control, Atlanta, Ga. naturally occuring penicillin-resistant strains exhibiting minimal inhibitory concentrations (MICs) of penicillin as high as 2 μg/ml. Since the relationship between blood concentration and minimal bactericidal concentration for the infecting gonococcus is reflected in the time required to affect a bactericidal response at the focus of infection, not only is the antibiotic level and its clearing time important but also the level of susceptibility of the gonococcus to the penicillin (28). In 1964, Thayer and Moore published findings showing that gonococci in vitro could survive and multiply in concentrations of penicillin below their minimal bactericidal concentrations (27). Thus, the opportunity for survival of more resistant strains is evident.

Resistance to a β-lactam antibiotic may be occasioned by a β-lactamase which converts penicillin G to the antibiotically inactive benzyl penicilloic acid, although there is not universal agreement that β-lactamase is involved in resistance to penicillin in either gram-positive or gram-negative cells (10, 12, 23), or by an acylase which converts the penicillin to 6-amino penicillanic acid, a product exhibiting at best 1/1,000 the activity of the parent molecule. The acylase, though theoretically possible as an explanation of resistance to penicillin, is probably not a factor in decreased susceptibility to the antibiotics since bacterial acylases have low affinities for their substrates. Although deacylation occurs rapidly in the cell-free state where concentrations of substrates are readily controlled in vivo, concentrations in whole cells are probably so low as to reduce activity markedly (11). Further, bacterial acylases are inhibited by therapeutically achieved levels of some penicillins (13, 26). In addition, biosynthesis of the enzyme is inhibited by temperatures above 31 C. Perhaps, more cogently, it has been shown in a study of 148 clinical isolates that acylase played little or no role in in vitro resistance to penicillin (4).

Differential binding of penicillin in susceptible and resistant strains resulting from a cell

envelope alteration or permeability barrier to the antibiotic has also been considered as a mechanism of resistance. Over the last 25 years, there have been studies dealing with the uptake of radioactive penicillin by various bacterial genera and species. These investigations will be considered below in relation to the findings reported herein.

MATERIALS AND METHODS

Organisms. Various isogenic mutants of the penicillin-sensitive *N. gonorrhoeae* 39W (kindly supplied by H. Schneider, Walter Reed Army Institute of Research, Washington, D.C.), made resistant by passage in penicillin-containing medium, had MICs ranging between 0.9 and 12 μg/ml. The isogenic mutants retained their oxidase characteristics and sugar fermentation patterns.

Naturally occurring clinical isolates with susceptibilities varying between 0.008 and 2.0 μg/ml were kindly supplied by the Center for Disease Control, Atlanta, Ga. All organisms were morphologically and biochemically identified as *N. gonorrhoeae*.

Growth of the organism. (i) Liquid media. The microorganisms were grown on defined GC liquid medium plus supplement as described by Kellogg et al. (14) with the following modifications: 10 ml of 4.2% $NaHCO_3$ and 10 ml of 10% soluble starch were added per liter to the medium post-sterilization. The supplement, also added after autoclaving, further contained 83 mg of $Fe(NO_3)_3 \cdot 9H_2O$, and 1 g of glutamine was used instead of 0.5 g/100 ml.

(ii) Solid media. Organisms were plated on GC agar base (Difco) supplemented with 10 ml of defined supplement per liter and grown for 18 h at 37 C in a candle jar.

Eighteen-hour-old cultures from solid media were scraped with a sterile platinum loop and suspended in liquid media to an optical density at 650 nm (OD_{650}) of 0.18 and grown at 37 C on a gyratory platform shaker (New Brunswick Scientific) revolving at 180 rpm.

[^{14}C]potassium benzyl penicillin with specific activity of 28.6 mCi/mmol was obtained from Amersham Searle.

β-Lactamase determination. The timed iodometric method of Citri (2) was used for β-lactamase determination. Eighteen-hour-old cultures were harvested from solid media, resuspended in liquid media to an OD_{650} of 0.18 and grown to at least double their original density when a 10-ml aliquot was withdrawn and centrifuged at 12,000 × *g* for 10 min. The resulting pellet was resuspended in 0.8 ml of fresh liquid media and exposed to inducing levels of penicillin G (1 μg/ml, final concentration). A control containing water in lieu of antibiotic was run concomitantly. The final OD_{650} of the suspension approximated 2.0. The cells were then shaken for 2 to 3 h in a reciprocal incubator shaker at 37 C and assayed for evidence of penicillinase activity.

Release of periplasmic penicillinase. The method of Neu (19, 20) was used for release of periplasmic penicillinase. Cells were grown in liquid medium to OD_{650} of 0.6 or greater and centrifuged at 12,000 × *g* for 10 min. The cells were washed three times with 0.85% NaCl at 3 C. An aliquot was saved for sonic treatment. The final pellet was suspended in 0.5 M sucrose containing 0.03 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.5) at 21 C in a ratio of 250 mg of wet cells to 20 ml of sucrose-tris-(hydroxymethyl)aminomethane. Ethylenediaminetetraacetic acid was added to a concentration of 1 mM. After 2 to 10 min of shaking, the cells were centrifuged at 4 C at 12,000 rpm for 10 minutes, resuspended in the same volume of distilled water, dispersed for 5 min at 3 C in a Vortex mixer, and then pelleted at 12,000 × *g* for 10 min. All washes and cells disrupted in the Branson 20K ultrasonic disintegrator for 1 to 3 min were assayed for penicillinase activity directly as well as after 10- to 20-fold concentration. Viability of cells was determined by plating, and spheroplast formation was observed under phase microscopy.

Amidase determination. Eighteen-hour-old cells were grown in liquid medium until an OD_{650} of 0.5 was reached. Twenty milliliters of this culture was centrifuged, and the pellet was suspended in 2.0 ml of 0.1 M phosphate buffer, pH 7.5, containing sufficient potassium benzyl penicillin to yield a final concentration of 20 mg/ml. The reaction mixture was incubated in a water bath at 37 C with intermittent shaking for 4 h and then centrifuged at 5,000 × *g*. The supernatant was assayed for the presence of 6-aminopenicillanic acid (1, 3).

Binding of radiolabeled penicillin. Lyophilized [^{14}C]penicillin was diluted to yield approximately 207 μg/ml with a specific activity of 15.7 μCi/ml. Overnight cultures of *N. gonorrhoeae* on solid medium were suspended in 100 ml of liquid media to an OD_{650} of approximately 0.180 and grown in a platform shaker at 37 C until an OD_{650} of 0.5 was attained. At this time, the cells were centrifuged, and the pellet was resuspended in 5 ml of Gey buffered saline salts to give a concentration of 10^{10} cells/ml. Cells were then exposed at 37 C to [^{14}C]penicillin at a final concentration of 4 μg/ml for up to 1 h. Petroff-Hauser chamber and viability counts were performed at 0, 30 and 60 min. At zero time, 1 ml of the suspension was removed and centrifuged at 12,000 × *g* for 10 min and the pellets were washed three times in Gey buffered saline salt. The first-, second-, and third-wash supernatants with buffered saline salt and the pellet were counted in a Beckman LS150 scintillation counter (in a scintillation fluid composed of 80 ml of Permafluor [Beckman], 1,920 ml of toluene and 1,000 ml of Triton X-100). Each sample was counted in duplicate for 20 min.

RESULTS

Enzyme determination. Table 1 shows 15 naturally occurring strains, two-thirds of which are relatively resistant to penicillin. The other five strains are exquisitely susceptible to the antibiotic. Four other strains, three of them isogenic mutants made resistant from the parent by serial stepwise passage in increasing concentration of penicillin, are listed.

The results indicated in this table rule out the

TABLE 1. *MIC of strains tested for penicillinase and amidase*[a]

Strain	MIC (μg/ml)	Strain	MIC (μg/ml)	Strain	MIC (μg/ml)	Strain	MIC (μg/ml)
GC 39-Wed	0.008	F13	0.008	F16	0.25	F18	0.5[b]
GC 39-1.5	0.9	F15	0.008	F17	0.25	F19	1.0[b]
GC 39-5	3.0	37492	0.008	43137	0.125	33684	1.0
GC 39-20	12.0[b]	43140	0.008	43138	0.125	62168M	2.0[b]
		62160	0.008	62162	0.25	62169	1.0

[a] No penicillinase or amidase was demonstrated under regular assay methods.
[b] No penicillinase was demonstrated after osmotic shock or after sonic disruption of cells.

presence of measurable β-lactamase activity of either a constitutive or inducible nature. (Both benzyl penicillin and methicillin were used as inducers.) Furthermore, the possibility of the enzyme being in the periplasmic space or culture filtrate was ruled out, as osmotically shocked cells failed to show β-lactamase activity. In addition, lack of permeability to penicillin as a mechanism of resistance in this system was ruled out, as sonically treated cells also showed no β-lactamase. No amidase (penicillin acylase) activity was observed in the same strains.

[^{14}C]penicillin binding. Table 2 depicts the binding of [^{14}C]penicillin by naturally occurring strains of *N. gonorrhoeae* in typical experiments. Three of the strains are very susceptible to the antibiotic on the basis of MIC (0.008 μg/ml). The rest of the strains can be catalogued (again on the basis of MIC) into moderately resistant, with MIC ranging up to 0.25 μg/ml, and resistant, with MIC up to 2.0 μg/ml. The binding of [^{14}C]penicillin molecules per cell was determined at 30 and 60 min. Each result was repeated several times. The binding at 30 min is reported since those at 60 min were essentially similar to those at 30 min. The first group defined as susceptible bound 3,200 to 3,800 molecules of [^{14}C]benzyl penicillin per cell. This is in definite contrast to the moderately resistant and resistant cells in which binding of [^{14}C]penicillin molecules ranged from 530 in strain F17 to 1,500 in strain 62162. An internal correlation between the binding of the moderately resistant and resistant strains and their MIC was not readily apparent. All molecules were firmly bound and failed to wash out after repeated attempts in buffer. All susceptible strains tested bound significantly higher levels of the antibiotic at 30 min than all less susceptible strains. Quench curves were made to insure reproducibility and accuracy of the results.

TABLE 2. *Molecules of* [^{14}C]penicillin bound per cell at 30 min

Strain	MIC (μg/ml)	Molecules/cell
37492	0.008	3,810
F15	0.008	3,540
GCW	0.008	3,200
Moderately resistant		
F17	0.25	530
43137	0.125	585
43138	0.125	750
62162	0.25	1,500
F18	0.5	1,150
33684	1.0	970
62168M	2.0	660
62169	1.0	1,000

Specificity of [^{14}C]penicillin binding. Table 3 presents data designed to show specificity of [^{14}C]penicillin binding by susceptible and resistant strains of *N. gonorrhoeae*. For this purpose two extremes in the collection were tested, first in their binding of [^{14}C]penicillin and then in their binding of [^{14}C]penicilloic acid under similar conditions. Cells (10^{10}) of strain 62168 M with an MIC of 2 μg/ml bound 670 molecules of [^{14}C]penicillin/cell at 30 min, whereas the same strain bound only 390 molecules of [^{14}C]benzyl penicilloic acid at 30 min. On the other hand, F15 sensitive to 0.008 μg of penicillin per ml bound 3,540 molecules of [^{14}C]penicillin/cell and 400 molecules of [^{14}C]penicilloic acid at identical times. Despite the obvious difference in binding the intact [^{14}C]penicillin molecules, once the β-lactam was hydrolyzed with a

TABLE 3. *Specificity of* [^{14}C]penicillin binding at 30 min

Strain (10^{10} cells/ml)	MIC (μg/ml)	Molecules of ^{14}C bound/cell	^{14}C (post-penase) molecules bound/cell
62168 M	2.0	670	390
F15	0.008	3,540	400

β-lactamase, binding was apparently nonspecific and indistinct between the two strains (i.e., 390 molecules of [^{14}C]benzyl penicilloic acid in strain 62168 M and 400 molecules of [^{14}C]penicilloic acid in strain F15).

To secure more definitive indication of the site of binding of the penicillin, cytoplasmic membrane preparations were exposed to [^{14}C]penicillin. Once again an inverse relationship between MIC and penicillin bound was demonstrated (Table 4). Cell wall preparations bound the antibiotic minimally, if at all, since as viewed in the electron microscope walls were contaminated with membranes but not vice versa. It is interesting that in both strains, the "nonspecific" binding as measured by boiled membrane uptake represents 59% of the total penicillin bound. This is, of course, not surprising and is consistent with the well-known capacity of penicillin to bind to various proteins.

DISCUSSION

The failure to demonstrate enzymatic inactivation as an explanation for penicillin resistance in the gonococcus led us to consider differences in the binding of the antibiotic as a possible mechanism for resistance.

Rowley et al. (22), probably the first to use radiolabeled penicillin to measure uptake of the antibiotic, showed that under nongrowing conditions [^{35}S]penicillin was taken up by two penicillin-susceptible strains of *Staphylococcus aureus* at a rate which increased with increasing concentration of the antibiotic. The curve of uptake differed markedly when susceptible and resistant organisms were used. Susceptible *S. aureus* bound up to six to eight times as much as a function of concentration of the antibiotic as did resistant staphylococci. In 1967, Rogers (21) reported on the significance of the correlation between susceptibility and total binding of labeled penicillin by representative genera of bacteria. The organisms, when grown in 0.01 μg of penicillin G per ml, bound amounts which correlated inversely with the minimum lethal dose and MIC, i.e., resistant cells bound less penicillin than susceptible cells.

TABLE 4. *Nanomoles of [^{14}C]penicillin bound per gram of enzyme protein*[a]

Strain	MIC (μg/ml)	Penicillin G bound[b]
37492	0.008	72
62162	0.250	17

[a] The cytoplasmic membrane resulting from homogenization with glass beads and differential ultracentrifugation was exposed to 4 μg of ^{14}C/ml for 20 min, and after washing at 100,000 × g three times the pellet was counted. A "boiled membrane" suspension prepared by placing the cytoplasmic membrane preparation in a boiling water bath prior to exposure to [^{14}C]penicillin was treated similarly (25).

[b] Binding by boiled preparation subtracted 106 nmol in strain 37492 and 25 nmol in strain 62162.

Few et al. (9) reported that the pencillin was bound to a "cell wall preparation" in *S. aureus*, whereas little if any antibiotic binding capacity was detected in the nondialyzable fraction of the cytoplasm. Cooper (5) characterized the penicillin-binding component as a cell wall fraction in association with lipid particles (18) containing a large amount of protein. Eagle (7, 8) reported that bacteria exposed to [^{14}C]- or [^{35}S]penicillin bound and concentrated the antibiotic from low external concentrations and that the amount bound at such levels by wild-type, naturally occurring strains was proportional to their susceptibility to the antibiotic. Obviously, the correlation between MIC and [^{14}C]penicillin will be nonexistent in a penicillinase producer where the organism will not only fail to bind the antibiotic but will be resistant to the product of hydrolysis.

In 1964, Duerksen (6) reported that [^{35}S]penicillin was bound, in a penicillinase-inducible *Bacillus cereus*, to a complex "closely associated" with the cytoplasmic membrane-cell wall area. Since the complex after treatment with a lipase and trypsin became dialyzable, it was suggested that the penicillin binding site was a lipoprotein.

Lawrence and Strominger (15) reported that in *B. subtilis*, enzyme preparations from the cytoplasmic membrane bound labeled benzyl penicillin and further that the bound antibiotic is released on treatment of the complex with hydroxylamine or ethanethiol, with recovery of penicilloyl derivatives. Furthermore, in 1972, Suginaka et al. (25) reported that virtually all the binding sites of *B. cereus*, *B. subtilis*, *S. aureus* and *Escherichia coli* resided in the cell membrane, and it was observed that more than one binding site is present in all organisms, *E. coli* and *S. aureus* having, however, only a single major component as opposed to more than one in the bacilli. In contrast to the carboxypeptidase binding component, susceptibility of the transpeptidase paralleled the susceptibility of the cell to the antibiotic (24).

We have ruled out the presence of penicillinase or acylase in our strains. Our results indicate a correlation which points to the possibility of a permeability or exclusion barrier, as measured by changes in the binding site for a

possible mechanism of decreased susceptibility to penicillin of *N. gonorrhoeae*.

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