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Hypersusceptibility of Penicillin-Treated Group B Streptococci to Bactericidal Activity of Human Polymorphonuclear Leukocytes

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Pretreatment of serotype Ib group B streptococci with benzylpenicillin, other beta-lactam antibiotics, or vancomycin increased the susceptibility of these bacteria to the bactericidal activity of a mixture of human polymorphonuclear leukocytes and normal human serum. Increased susceptibility of the bacteria to killing by phagocytes was elicited even by exposure to subinhibitory levels of the beta-lactam antibiotics. Inhibitors of protein synthesis did not induce such susceptibility. We investigated the possible biochemical basis of penicillin-induced susceptibility to phagocytosis. Penicillin treatment induced the release of substantial quantities of group B streptococcal surface components into the growth medium (lipoteichoic acid, lipid, and capsular polysaccharide). Labeling of the live streptococci with ^{3}H-labeled penicillin was used to evaluate the effect of exposure to subinhibitory concentrations of this antibiotic on the penicillin-binding proteins. Our results suggested that beta-lactam antibiotics and components of the immune system may act in concert to eliminate invading bacteria.

Group B streptococci are important human pathogens, particularly in the neonatal period (2, 3, 10, 30, 31). Serotype Ib streptococci are most frequently associated with septicemia occurring in the first few days after birth (2, 10, 30, 31). Infections with these bacteria have high mortality rates, even with the prompt application of chemotherapy (4, 10). The antibiotic used most frequently to treat group B streptococcal infections is benzylpenicillin (7). Prophylactic use of penicillin for the eradication of group B streptococci from carriers has also been proposed by some workers (10), although this is considered impractical by others (3).

The primary host defense against this group of pathogens is antibody-mediated phagocytosis (1, 8, 13, 20, 21, 24, 25). It has been proposed that the high degree of susceptibility of neonates to group B streptococcal infection results from immature immune defenses (6, 25, 27). This suggestion, along with the recent upsurge of group B streptococcal infections in the United States, has stimulated programs to boost the immune system against these pathogens by vaccination (6).

In this paper, we report evidence suggesting that penicillin and components of the host immune system may cooperate in eliminating the invading bacteria. This may be another in the increasing number of observations indicating synergistic action between a chemotherapeutic agent and host defense factors (9, 11, 12, 17, 23, 28).

MATERIALS AND METHODS

Bacterial strains and growth medium. The group B streptococci used in this study were the rough strain 090R and strain H36B (serotype Ib) and were obtained from R. Lancefield, The Rockefeller University. Routinely, these bacteria were grown in Todd-Hewitt broth supplemented with glucose and sodium diphosphate as described by Baker and Kasper (5). Growth was monitored by using a Coleman nephelometer. Viable titers were determined with 4% sheep blood agar plates. Minimal inhibitory concentrations (MIC) were determined by a broth dilution method (17).

Antibiotics and reagents. ^{3}H-labeled benzylpenicillin (ethylpiperidium salt; 31 Ci/mmol) was the generous gift of A. Rosegay, Merck, Sharp & Dohme, Rahway, N.J. This antibiotic was stored at −20°C in acetone and diluted in 10 mM potassium phosphate buffer (pH 6.8) before use. Other unlabeled antibiotics used included benzylpenicillin, cephaloridine, and vancomycin from Eli Lily & Co., Indianapolis, Ind., cefoxitin from Merck, Sharp & Dohme, piperacillin from Lederle Laboratories, Pearl River, N.Y., cefadroxil and gentamicin from Bristol Laboratories, Syracuse, N.Y., methicillin from Beecham Laboratories, Piscataway, N.J., and chloramphenicol from Sigma Chemical Co., St. Louis, Mo. The M1 *N*-acetyl muramidase was kindly provided by K. Yokogawa, Dainippon Pharmaceutical Co., Suita/Osaka, Japan. The protease inhibitor phenylmethylsulfonyl fluoride was obtained from Sigma Chemical Co. Acrylamide and *N,N*-meth-

ylenebisacrylamide were electrophoresis grade (Bio-Rad Laboratories, Rockville, N.Y.). All other chemicals were reagent grade and were obtained commercially.

Human serum pool. Sera were prepared from the blood of healthy adult donors, pooled, and stored at −70°C in 1-ml samples. Complement was inactivated by heating at 56°C for 30 min. The serum pool was absorbed with whole bacteria (strain H36B), as described by Baltimore et al. (8).

Bactericidal activity of human PMN against serotype Ib group B streptococci. Human polymorphonuclear leukocytes (PMN) were obtained from heparinized blood drawn from healthy laboratory volunteers and were prepared by the dextran sedimentation method of Roberts (22). The pelleted PMN were suspended in Gey balanced salt solution to give a final concentration of 6×10^6 PMN/ml. Bacteria were grown exponentially with or without antibiotic for 1 h at 37°C, and then penicillinase (100 U/ml) was added; the bacteria were harvested, washed, and resuspended in balanced salt solution. The bacteria (final cell density, 2×10^6 colony-forming units [CFU] per ml) were mixed with the PMN in the presence of 10% human serum. This preparation was tumbled with a tube rotator (Sepco, Baltimore, Md.) for 1 h at 37°C. Samples (0.1 ml) were removed at 0, 30, and 60 min and added to 0.9 ml of 0.01% bovine serum albumin in order to lyse the PMN. After vigorous blending with a Vortex mixer for 30 s, additional 10-fold dilutions were made in sterile saline for plating. Appropriate controls were included to rule out killing by the antibiotics or by the serum.

Biosynthetic labeling of lipids and lipoteichoic acid. The bacteria were labeled in Todd-Hewitt broth supplemented with [2-^{3}H]glycerol (1 μCi/ml; 1 μg/ml; New England Nuclear Corp., Boston, Mass.). The following three labeling regimes were used. (i) The radioactive glycerol was added to the culture simultaneously with the penicillin (designated new label). (ii) The glycerol was added to the culture when the cell density was 2×10^6 CFU/ml, and the bacteria were then incubated for 90 min before harvesting. The cells were suspended in isotope-free medium and incubated for another 30 min before penicillin was added (designated old label). And (iii) the isotope-labeled glycerol was added as described above for the old label but was left in the growth medium during penicillin treatment (designated continuous label). Incorporation into trichloroacetic acid-precipitable material (with bovine serum albumin as a carrier) was determined in total culture samples and supernatant fluid samples after 90 min of drug treatment at 37°C, as previously described (17).

Release of the type-specific polysaccharide from the type Ib strain. Cultures of strain H36B in 100 ml of Todd-Hewitt broth at a cell density of 8×10^7 CFU/ml were divided in half. One-half of each culture received one-third to three times the MIC of penicillin for 1 h at 37°C, and the other half served as a control. After the bacteria were removed by centrifugation, the resulting supernatant fluids were concentrated by pressure dialysis (PM10 membrane; Amicon Corp., Lexington, Mass.) and ethanol precipitation (80%, vol/vol) (26). The precipitates were dissolved in 500 μl of saline, and 5-μl samples were assayed for type-specific polysaccharide by rocket immunoelectrophoresis according to the method of Weeke (29) or by single radial immunodiffusion. The type-specific rabbit antiserum used in these procedures was kindly donated by J. Tai, The Rockefeller University.

Labeling of the penicillin-binding proteins of type Ib group B streptococci. To label penicillin-binding proteins (PBPs), 1-ml samples of exponentially growing bacteria (5×10^7 CFU/ml) were incubated with subinhibitory concentrations of ^{3}H-labeled penicillin or of nonradioactive penicillin at 37°C for 1 h. Binding of the radioactive penicillin was stopped by adding 5 μl of nonradioactive penicillin (300 μg). The cells were pelleted by centrifugation in an Eppendorf microcentrifuge at 8,000 × *g* for 5 min and then suspended in 35 μl of 10 mM potassium phosphate buffer containing 2 mM phenylmethylsulfonyl fluoride. At this point, the cultures that had been pretreated with cold penicillin were exposed to 0.5 μg of ^{3}H-labeled penicillin (saturating concentration). The bacteria were subjected to three cycles of freezing and thawing before lysis at 37°C with 10 μg of M1 muramidase (6 min), followed by 2.5 μl of 20% Sarkosyl NL97; 25 μl of Laemmli-Favre sample dilution buffer (19) and 5 μl of mercaptoethanol were added, and the samples were boiled for 3 min and then applied to a polyacrylamide slab gel.

Slab gel electrophoresis and detection of PBPs. Discontinuous sodium dodecyl sulfate-polyacrylamide slab gels were prepared as described by Laemmli and Favre (19). The concentrations of acrylamide and *N*,*N*-methylenebisacrylamide were 10 and 0.166%, respectively, in the separating gel and 5 and 0.083%, respectively, in the stacking gel. The techniques used for electrophoresis and detection of the PBPs by fluorography have been described previously (32). The fluorograms were exposed to the gels for 10 days at −70°C.

RESULTS

Sensitization of type Ib group B streptococci to phagocytosis by human PMN. Table 1 shows that increased susceptibility of strain H36B to killing by PMN was elicited by benzylpenicillin but not by chloramphenicol. The killing of both antibiotic-treated and untreated bacteria by the PMN apparently required both specific antibody and a heat-labile factor(s), presumably complement (Table 1).

In addition to benzylpenicillin, five other beta-lactam antibiotics (tested at one-half the MIC for 1 h) were also capable of sensitizing streptococci to phagocytosis (Fig. 1 and Table 2). Although there were wide variations in the results of the phagocytic bactericidal assays in these experiments (Table 2), the increase in susceptibility induced by the beta-lactam antibiotics and by vancomycin was fairly constant. The sensitizing activity of benzylpenicillin could be detected at a concentration as low as one-eighth the MIC (Fig. 2). Vancomycin was an active sensitizing

TABLE 1. *Killing of antibiotic-pretreated serotype Ib group B streptococcal strain H36B by human PMN*

Antibiotic[a]	% Survival after 1 h[b]			
	PMN plus normal human serum	PMN plus absorbed serum	Normal serum alone	PMN plus heated serum
None	0.81 (0.45)	Growth	Growth	Growth
Chloramphenicol	1.02 (0.30)	77.5 (12.5)	100	100
Benzylpenicillin	0.056 (0.03)	30.8 (20.1)	62.8 (20.6)	54.6 (17.8)

[a] The bacteria were treated in Todd-Hewitt broth for 1 h at 37°C with the MIC of penicillin or of chloramphenicol, harvested, and washed with balanced salt solution before exposure to PMN.

[b] The results are expressed as percentages of the zero-time sample surviving after 1 h. The values are the mean values for five determinations. The numbers in parentheses are standard deviations of the mean.

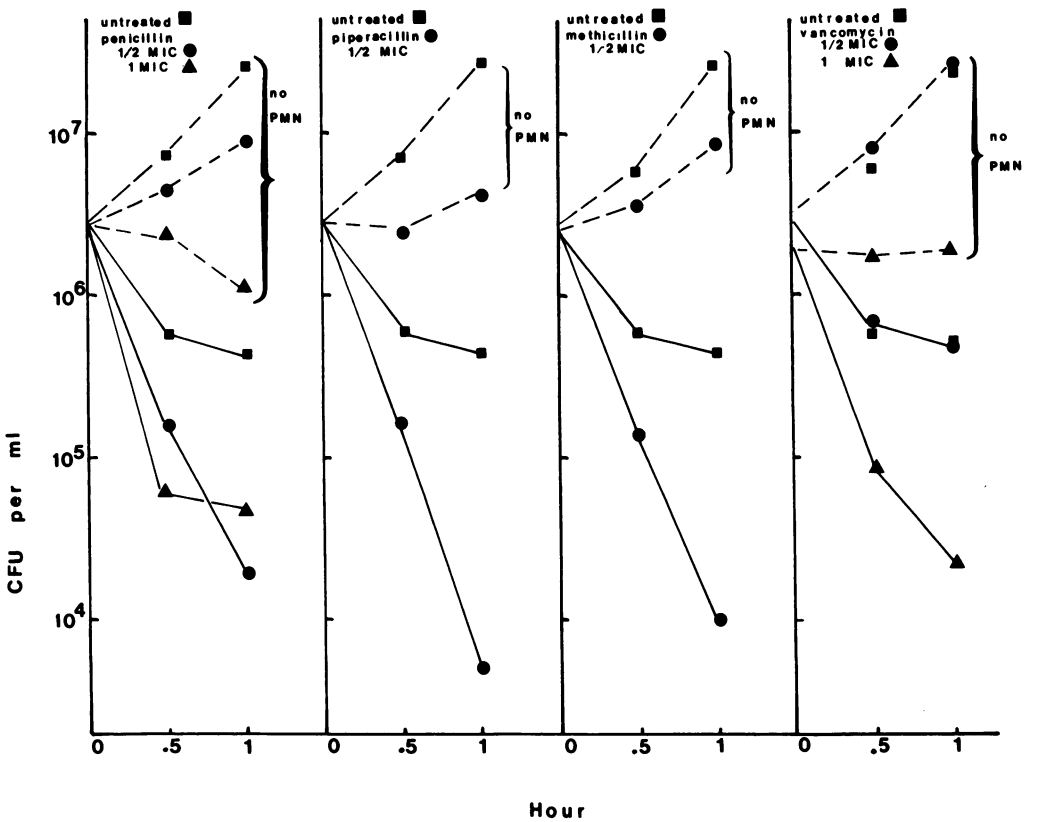

FIG. 1. *Killing of serotype Ib group B streptococci (strain H36B) by human PMN: effects of pretreatment with inhibitors of peptidoglycan synthesis. The MICs were 0.03, 0.2, 0.7, and 1.5 μg/ml for penicillin, piperacillin, methicillin, and vancomycin, respectively.*

agent at the MIC but not at one-half the MIC (Table 2, and Fig. 1). Although the degree of sensitization by vancomycin was comparable to that of benzylpenicillin, the in vitro bactericidal activity of vancomycin was much lower than the activities of penicillin (Fig. 3) and the other beta-lactam antibiotics (data not shown). Sensitization to phagocytosis was not apparent with antibiotics that did not inhibit cell wall synthesis (Fig. 4). Pretreatment with gentamicin or chloramphenicol failed to accelerate the killing by PMN. Simultaneous treatment with chloramphenicol noticeably decreased the sensitization by penicillin (particularly after 30 min of incubation with the PMN). Although limited, this decrease was reproduced several times. Gentamicin had no such antagonistic effect. This finding correlates with the other in vitro effects of

TABLE 2. *Killing of serotype Ib group B streptococcal strain H36B by human PMN: effect of pretreatment with antibiotics*

Antibiotic[a]	Concn (μg/ml)	Fraction of MIC	% Survival after 1 h[b]		
			Untreated + PMN[c]	Antibiotic treated + PMN	Antibiotic treated − PMN
Cephaloridine	0.007	0.5	23.7[d]	6.32	>100
	0.007	0.5	13.2[d]	0.80	69
Benzylpenicillin	0.015	0.5	23.7	0.62	>100
	0.015	0.5	13.2	0.68	>100
	0.015	0.5	19.9	0.40	60
	0.030	1	10.5	1.80	87
Piperacillin	0.10	0.5	23.7	0.58	>100
	0.10	0.5	13.2	0.21	>100
Methicillin	0.35	0.5	23.7	1.26	>100
	0.35	0.5	13.2	0.47	100
Cefadroxil	0.8	0.5	13.2	0.02	86.5
	0.8	0.5	4.9	0.10	72.0
Cefoxitin	2.5	0.5	23.7	5.36	>100
	2.5	0.5	13.2	0.80	52
Vancomycin	0.7	0.5	13.2	16.2	>100
	1.5	1	13.2	0.1	100
	1.5	1	11.2	1.16	100
	1.5	1	4.9	0.20	74
Gentamicin	14.0	0.5	19.9	22.1	>100
	28.0	1	10.8	3.4	100
Chloramphenicol	3.0	1	10.5	15.90	100
	3.0	1	19.9	6.4	100

[a] Bacteria were pretreated for 1 h at 37°C with an antibiotic in Todd-Hewitt broth before exposure to the PMN plus normal human serum.
[b] Results are expressed as percentages of the zero-time sample surviving after 1 h.
[c] Survival of all untreated samples after 1 h without PMN was 100% or more.
[d] Separate determinations on different days.

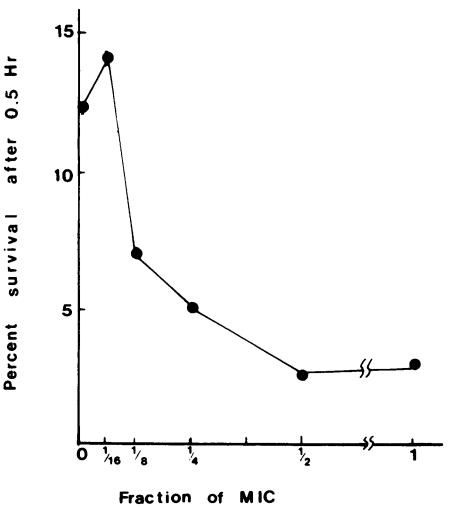

FIG. 2. *Sensitization of serotype Ib group B streptococci (strain H36B) to the bactericidal action of human PMN by pretreatment with subinhibitory concentrations of penicillin for 60 min at 37°C. The data are expressed as percentages of bacteria surviving after they were mixed with PMN for 60 min at 37°C.*

chloramphenicol and gentamicin; chloramphenicol interferes with the bactericidal action of penicillin (18), whereas gentamicin increases it (data not shown).

Release of cell surface components from group B streptococci. In previous reports, we described a newly recognized consequence of penicillin treatment in autolysis-defective bacteria (pneumococci and group A and H streptococci), namely, the massive release of cell surface polymers, including newly synthesized cell wall polymers and membrane-associated lipoteichoic acids and lipids (14–16). It is possible that alterations of the cell surface due to the loss of specific polymers may have been involved in the sensitization to phagocytosis. As Table 3 shows, this finding has been extended to group B streptococci. Biosynthetic labeling of either strain 090R or strain H36B with [2-^{3}H]glycerol indicated that penicillin (at 10 times the MIC) stimulated the release of macromolecular glycerol, regardless of the biosynthetic age of the polymers (Table 3). Further analyses of the released material by various extraction procedures, column chromatography, and passive hemaggluti-

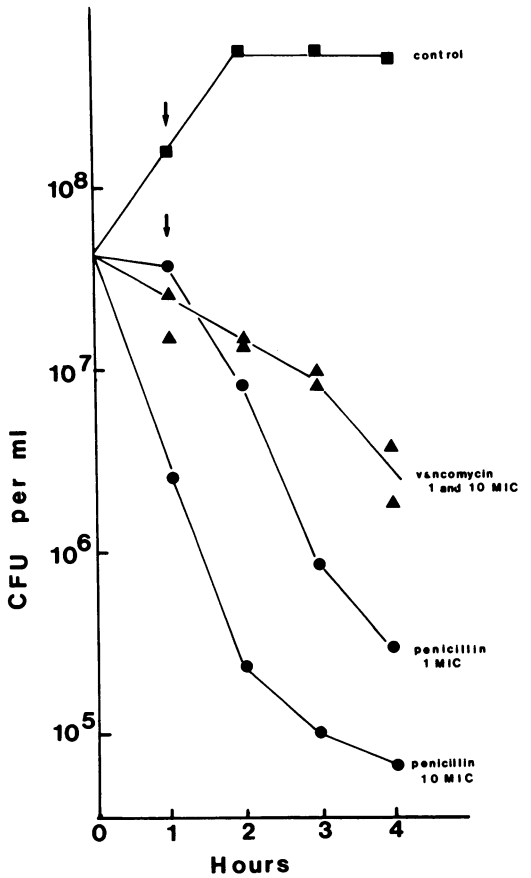

FIG. 3. *In vitro bactericidal activities of vancomycin and penicillin against group B serotype Ib streptococci (strain H36B) in the early exponential phase of growth. The arrows indicate the times when samples were removed for phagocytic assays (see Fig. 1 for the results of the phagocytic tests).*

nation, as described previously (15, 16), revealed the presence of cellular lipids, lipoteichoic acid, and, possibly, deacylated lipoteichoic acid (data not shown).

We also investigated the possible release in the presence of penicillin of another surface polymer, the type-specific capsular polysaccharide. This was of interest because the type-specific antigens of group B streptococci are presumed to be antiphagocytic (1, 8, 20, 24). Penicillin present at three times the MIC for 1 h at 37°C induced an apparent twofold stimulation in the release of the capsular antigen(s) (Fig. 5A). An analysis of the cell-free material by single radial diffusion also showed significant stimulation of capsular antigen release by a concentration of penicillin as low as one-third the MIC (Fig. 5B).

Sensitization of "conditionally" penicillin-tolerant group B streptococci. Recently, it was found that growth of group B streptococci in media adjusted to initial pH values below 6 renders the bacteria tolerant to the bactericidal effects of penicillin (Horne and Tomasz, manuscript in preparation), even at concentrations greater than the MIC. However, the growth of such bacteria is still inhibited by the antibiotic. We wanted to determine whether the low-pH conditions that interfered with the bactericidal action of penicillin also blocked the sensitization to phagocytosis at subinhibitory concentrations of the antibiotic. However, bacteria grown in medium adjusted to pH 5.5 rather than in the normal medium (pH 7.2 to 7.4) still exhibited increased susceptibility to the PMN after penicillin treatment (Table 4).

Investigation of the binding of penicillin to the PBPs of group B streptococci. The binding of penicillin to the PBPs of bacteria under conditions identical to those used for the sensitization (i.e., subinhibitory concentrations for 1 h at 37°C) was determined in two ways; either ^{3}H-labeled penicillin was added directly to the cultures, or nonlabeled penicillin was bound first to the cells in the medium and then a saturating amount of radioactive penicillin was added to the cells after they were harvested and resuspended in buffer. The labeled antibiotic was added to whole cells in buffer to prevent continued synthesis of the PBPs. In this manner, we tried to determine the quantities of the PBPs available for binding after prolonged treatment with subinhibitory concentrations of penicillin.

Figures 6A and 6B show the results of these studies. Both types of experiments indicated that substantial amounts of penicillin were bound to all of the PBPs (with the exception of PBP 2, which was a minor band) at concentrations as low as one-fifth to one-tenth the MIC. These concentrations were within the range of concentrations causing increased susceptibility of the bacteria to killing by PMN. Penicillin concentrations one-fifth and one-tenth the MIC had no discernible effect on the growth of the bacteria (data not shown).

DISCUSSION

The basic finding reported here is that serotype Ib group B streptococci treated with subinhibitory concentrations of inhibitors of peptidoglycan synthesis are hypersensitive to the bactericidal activity of human neutrophils (PMN). At concentrations below the MIC, all six of the beta-lactam antibiotics used in this study were capable of increasing the susceptibility of group B streptococci to the bactericidal action of hu-

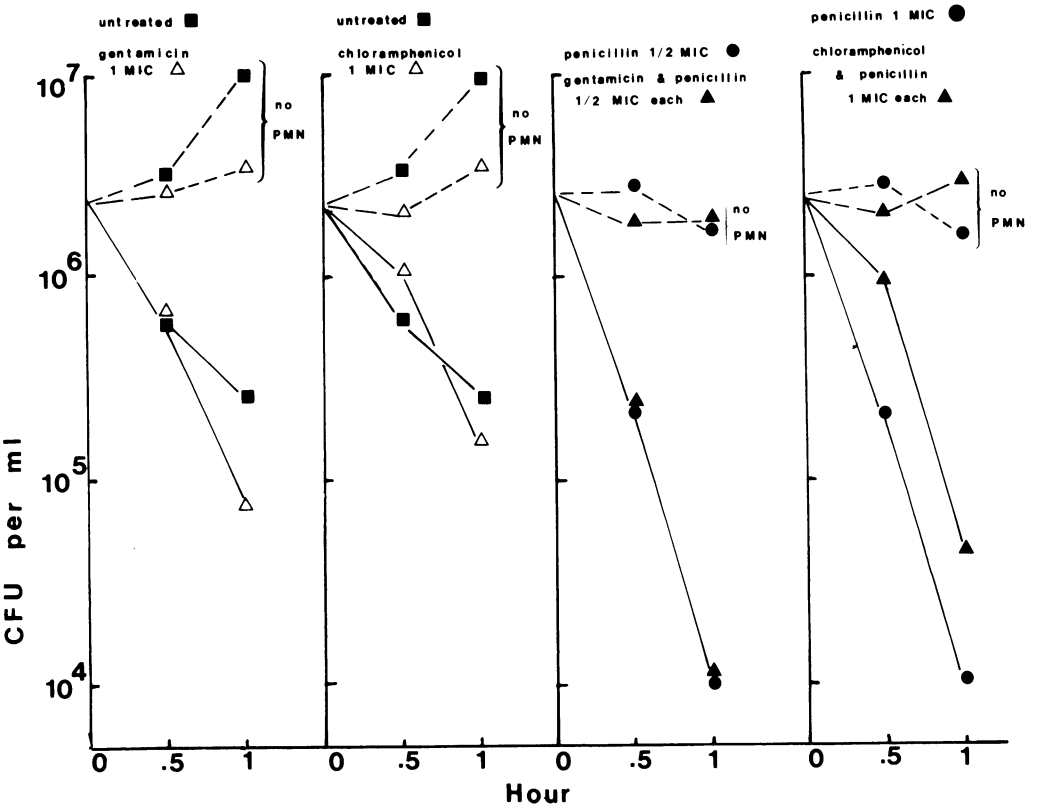

FIG. 4. *Killing of serotype Ib group B streptococci (strain H36B) by PMN: effect of pretreatment with gentamicin or chloramphenicol (alone or in combination with penicillin). The MICs were 0.03, 3.0, and 28 μg/ml for penicillin, chloramphenicol, and gentamicin, respectively.*

TABLE 3. *Secretion of macromolecular [^{3}H]glycerol from group B streptococci treated with penicillin*

Strain	Labeling regime[a]	Concn of benzyl-penicillin (×MIC)[b]	% Total incorporation (% of control)	% Secretion (% of total incorporation)
090R	New	0	100	2.1
		10	56	65.6
	Old	0	100	2.4
		10	68	59.4
H36B (Ib)	Continuous	0	100	5.0
		10	43	68.0

[a] Bacteria were labeled in Todd-Hewitt broth with [2-^{3}H]glycerol (1 μCi/ml; 1 μg/ml) by three different labeling regimens (see text): (i) radioactive glycerol was added to the culture together with penicillin (new label); (ii) the culture was labeled before the exposure to the antibiotic (old label); or (iii) the bacterial culture received the radioactive glycerol before the antibiotic (as in the old label method), but the cells were left in the isotope-containing medium throughout the penicillin treatment (continuous label). Incorporation into trichloroacetic acid-precipitable material with bovine serum albumin as a carrier was determined after 90 min of drug treatment at 37°C.

[b] The MIC of benzylpenicillin was 0.03 μg/ml for both strains.

man PMN. Vancomycin, another inhibitor of peptidoglycan synthesis, was also an active sensitizing agent at its MIC. No sensitization was elicited by inhibition of protein synthesis by treatment with gentamicin or chloramphenicol (both added at the MIC). In addition, gentamicin had no effect on penicillin-induced sensitization, whereas chloramphenicol had an antagonistic effect.

The mechanism of this sensitization is not

clear, but it may be due to penicillin-stimulated loss of capsular material or to some other alteration of the cell surface caused by the loss of membrane components, lipoteichoic acids, and lipids. These changes in the cell surface may facilitate the uptake of bacteria by PMN. Alternatively, these penicillin-induced changes could make the killing of bacteria internalized by PMN easier.

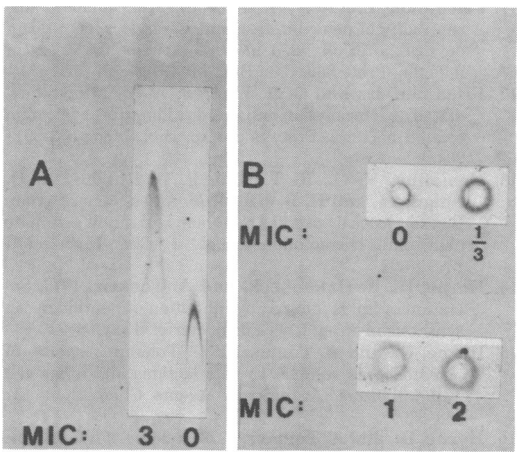

FIG. 5. *Release of the type-specific polysaccharide from group B streptococci after treatment with penicillin. Supernatant fluids were processed as described in the text. Samples (5 μl) were applied to wells in agarose and subjected to rocket immunoelectrophoresis (A) or to single radial immunodiffusion (B). The samples of supernatant fluids were from an untreated culture (well 0) and from cultures treated with penicillin at one-third the MIC, the MIC, two times the MIC, and three times the MIC.*

TABLE 4. *Killing of serotype Ib group B streptococcal strain H36B by PMN: effect of pretreatment with penicillin in low-pH (5.5) and high-pH (8.3) media*

pH of growth medium[a]	% Survival after 1 h of incubation with PMN[b]	
	Untreated	Pretreated with penicillin
5.5	1.26 (0.43)	0.19 (0.075)
8.3	5.81 (1.5)	0.69 (0.54)

[a] The bacteria were grown at 37°C in medium adjusted to pH 5.5 with 1 N HCl and to pH 8.3 with 1 N NaOH. At a cell density of 4×10^7 CFU/ml, benzylpenicillin (one-half the MIC) was added to one-half of the culture. Incubation was continued for another 1.25 generations (60 and 38 min at pH 5.5 and 8.3, respectively) before exposure to the PMN and normal human serum.

[b] The results are expressed as the percentages of the zero-time sample surviving after 1 h. The mean values for three determinations are shown, and the numbers in parentheses are the standard deviations of the mean.

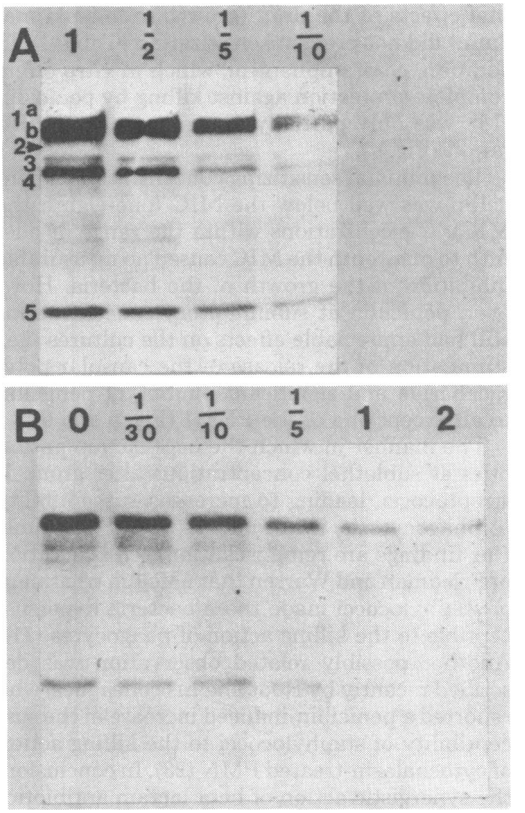

FIG. 6. *PBPs of group B streptococci. Exponentially growing cultures of strain H36B were treated with different concentrations of either ^{3}H-labeled penicillin (A) or nonradioactive penicillin followed by a saturating amount of radioactive penicillin (added to the cells in buffer) (B). The PBPs are numbered in order of decreasing molecular weight, as follows: bands 1a and 1b (molecular weight, 86,000), band 2 (80,000), band 3 (76,000), band 4 (71,000), and band 5 (42,000). The numbers at the tops of the columns indicate the fractions of the penicillin MIC added to the cultures. The MIC of radioactive penicillin was 0.05 μg/ml.*

It should be mentioned that the killing of bacteria by PMN required specific antibody and heat-labile factor(s) in the serum even after pretreatment with penicillin since prior absorption of the serum with live bacteria (4°C, 1 h) or heat inactivation (56°C, 0.5 h) prevented significant killing by PMN (Table 1).

It is possible that the sensitization phenomenon might be related to the bactericidal effects of the antibiotics. For example, vancomycin, unlike the beta-lactam antibiotics, was not active at subinhibitory concentrations and was also a rather poor bactericidal agent (Fig. 1 and 3). Treatment of bacteria with penicillin under conditions which offered protection against the le-

thal effects of the drug (growth in low-pH medium) did not prevent sensitization (Table 4). In addition, chloramphenicol, which in vitro offers complete protection against killing by penicillin (18), was only partially antagonistic to sensitization (Fig. 4).

The minimal sensitizing concentration of penicillin was well below the MIC (one-eighth the MIC). Concentrations within the range of one-fifth to one-tenth the MIC caused no measurable inhibition of the growth of the bacteria. However, penicillin at subinhibitory concentrations still had appreciable effects on the cultures (i.e., stimulation of the release of the capsular polysaccharide and significant binding of penicillin to all except one of the PBPs) (Fig. 5 and 6).

The manner in which the beta-lactam antibiotics at sublethal concentrations alter group B streptococci, leading to increased susceptibility to phagocytosis, is not understood at this time. Our findings are reminiscent of the observation of Friedman and Warren that nafcillin treatment of staphylococci made these bacteria hypersusceptible to the killing action of phagocytes (11). Another possibly related observation was described recently by Root and his colleagues, who reported a penicillin-induced increase in the susceptibility of staphylococci to the killing action of cytochalasin-treated PMN (23). In conclusion, the synergistic action of beta-lactam antibiotics and antibody-mediated phagocytosis on group B streptococci may have clinical relevance.

ACKNOWLEDGEMENT

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