INFECTION AND IMMUNITY, Jan. 1994, p. 162–165
0019-9567/94/$04.00+0
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Vol. 62, No. 1

# Inactivation of *Escherichia coli* Penicillin-Binding Proteins by Human Neutrophils

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Received 27 May 1993/Returned for modification 9 July 1993/Accepted 22 October 1993

**Neutrophils use a variety of microbicidal mechanisms in their role as one of the primary arms of the human host defense system. We have previously observed that a cell-free system containing myeloperoxidase (MPO), one of the major components of the neutrophil's oxidative antimicrobial systems, inactivated microbial penicillin-binding proteins (PBPs), which mediate the formation of the peptidoglycan layer of eubacterial cell walls. This is a potentially important mechanism of MPO-mediated bacterial toxicity. Since numerous other microbicidal systems, both oxidative and nonoxidative, are used by whole neutrophils, we investigated the effect of intact neutrophils on *Escherichia coli* PBPs. Penicillin binding activity was progressively reduced by neutrophil exposure for all PBPs. Loss of penicillin binding activity correlated well with loss of microbial viability for almost all PBPs. Azide-treated neutrophils, MPO-deficient neutrophils, and chronic granulomatous disease neutrophils inactivated *E. coli* PBPs in a manner similar to that of normal neutrophils, suggesting that MPO-independent, and even oxygen-independent, microbicidal systems are also involved in inactivation of PBPs. PBP inactivation, an antimicrobial strategy used by β-lactam-producing molds (and now by physicians), may be an important microbicidal mechanism used by human neutrophils.**

Neutrophils (PMNs), one of the major participants in the human host defense system's response to invading microorganisms, have a wide variety of oxidative and nonoxidative microbicidal pathways (8). Myeloperoxidase (MPO), an enzyme found in the azurophil granules of neutrophils, is a major component of the oxygen-dependent antimicrobial systems (7), and is active against a wide variety of pathogens, including bacteria (6), fungi (2), and viruses (1, 9).

Microbial penicillin-binding proteins (PBPs) are targets for attack by oxidants generated in a cell-free MPO-dependent microbicidal system (13). PBPs mediate the formation and remodeling of peptidoglycan cell wall components in all eubacteria (4, 18, 20). Inactivation of PBPs by β-lactam antibiotics disrupts balanced PBP activity and results in bacterial death. MPO treatment of *Escherichia coli* resulted in loss of penicillin binding by PBPs, strongly suggesting PBP inactivation. The rapidity of inactivation of certain essential PBPs, which correlated with loss of viability, along with *E. coli* morphologic changes consistent with this effect, led us to conclude that PBP inactivation could be a major mechanism of MPO-mediated bacterial toxicity. The microbicidal milieu within the PMN phagosome, with the possible participation of multiple microbicidal systems, is considerably more complex than the cell-free MPO system. Accordingly, we wished to determine the effects of intact PMNs on *E. coli* PBPs and the particular role of MPO and other oxygen-dependent systems in this process.

## MATERIALS AND METHODS

**Special reagents.** Reagents were obtained from Sigma Chemical Co., St. Louis, Mo., except benzyl penicillin *N*-ethyl piperidine salt, phenyl-$^3$H label, and En$^3$Hance (Du Pont, Wilmington, Del.).

**Bacteria.** *E. coli* (ATCC 11775; American Type Culture Collection, Rockville, Md.) cells were maintained on blood agar plates, grown overnight in Trypticase soy broth, and then diluted 100-fold and grown for 4 h. Organisms were harvested in log phase, washed twice, and suspended in Hanks' balanced salt solution with 1 mM calcium and 1 mM magnesium to the required $A_{540}$.

**Neutrophil isolation.** PMNs were isolated from EDTA-anticoagulated blood by dextran sedimentation, Ficoll-Hypaque centrifugation, and hypotonic lysis of residual erythrocytes (15). Cells were suspended in Hanks' balanced salt solution without calcium or magnesium at approximately 2.5 $\times$ $10^7$ PMNs per ml. Cells were ≥95% neutrophils by Diff-Quick staining, and viability was ≥96% by trypan blue exclusion. In some experiments, because of the large number of cells required, PMNs from two to three normal donors were combined. Chronic granulomatous disease (CGD) PMNs were isolated from a patient with a variant X-linked form of CGD in which a Pro→His substitution in gp91$^{phox}$ results in a mutant cytochrome $b_{558}$ (3, 12). MPO-deficient PMNs were isolated from a patient with a complete hereditary deficiency of MPO (14).

**PMN microbicidal system.** *E. coli* cells ($10^8$ CFU/ml) were incubated with PMNs (2 $\times$ $10^7$/ml) and 10% serum in Hanks' balanced salt solution in final volumes of 10 ml and tumbled at 8 RPM in 50-ml tubes at 37°C. For experiments with azide-inhibited PMNs, 5 mM sodium azide was included in the killing mixture. At the times indicated in the figure legends, 100 μl of 10% Triton X-100 was added and the mixtures were vortexed for 30 s to lyse PMNs. Approximately 40% of the bacterial inoculum could be recovered immediately after dilution into a PMN suspension. Triton X-100 (0.1%) did not affect *E. coli* viability. A 20-μl aliquot was diluted 1:10 in water for 10 min and serially diluted, and viability was determined by the pour plate method with

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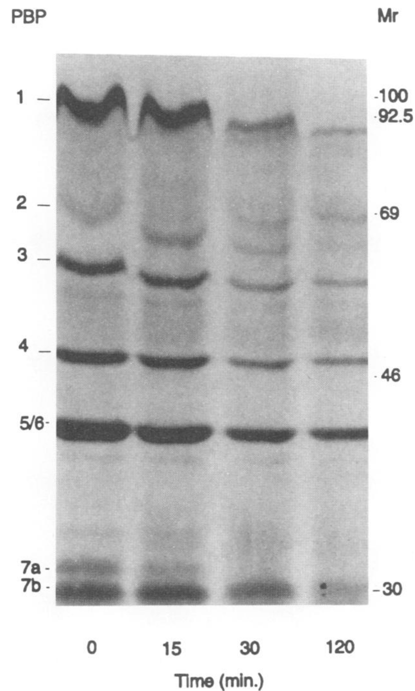


FIG. 1. Effect of human neutrophils on *E. coli* PBPs. *E. coli* cells were exposed to PMNs and 10% serum, and at indicated intervals, samples were labelled with $^3$H-benzyl penicillin and fluorographed as described in Materials and Methods. PBPs are numbered to the left according to Spratt (17), mobilities of molecular size markers are indicated to the right, and time of incubation is on the bottom.

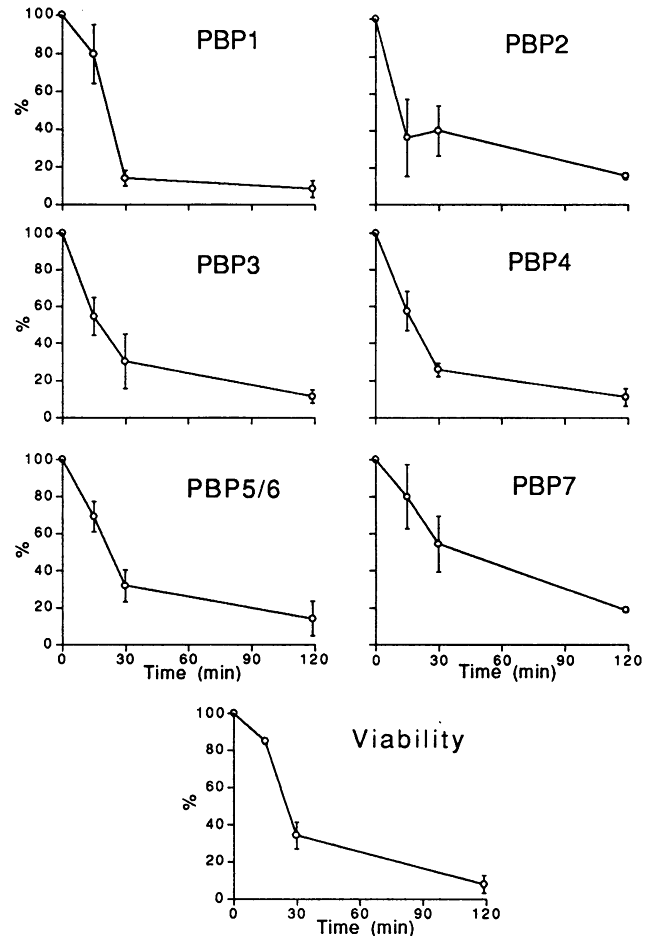


FIG. 2. Inhibition of *E. coli* penicillin binding by neutrophils. PBP binding activity and microbial viability were expressed as the percentage (mean ± standard error; $n$ = 3 to 5) of the integrated band intensity or the CFU per milliliter, respectively, at 0 min. *E. coli* viability at 0 min = (9.6 ± 0.8) × $10^7$ CFU/ml.

Trypticase soy agar. The remainder of the mixture was centrifuged at 450 × $g$ for 5 min, pelleted PMNs and debris were discarded, and the supernatant was spun at 11,000 × $g$ for 10 min. Pelleted *E. coli* cells were washed with 30 mM Tris, pH 8.0, and stored at −80°C.

**PBPs.** PBPs in bacterial membranes were detected as described previously (13). Briefly, thawed *E. coli* cells were treated with 5 mg of lysozyme per ml and 10 mM K-EDTA and then incubated with DNase, RNase, aprotinin, leupeptin, and soybean trypsin inhibitor, all at 10 μg/ml, and 20 mM $MgSO_4$. Membranes were collected by centrifugation at 50,000 × $g$ for 60 min at 4°C and suspended in 50 mM sodium phosphate, pH 7.0, to a protein concentration of 10 to 20 μg/ml (5). Membranes were incubated with 5 μg of benzyl penicillin *N*-ethyl piperidine salt–phenyl-$^3$H label for 10 min at 37°C. Sample buffer (11) was added, and samples were boiled for 5 minutes. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed with 8% running gels and 4% stacking gels, at 50 V for 15 h, with 250 to 450 μg of protein per lane (protein content was equivalent on all lanes of any one gel). Gels were stained with Coomassie blue and prepared for fluorography with En$^3$Hance. Dried gels were placed next to preflashed Kodak X-Omat AR-2 film at −80°C for 9 to 10 weeks. Densitometry was performed with a video densitometry and analysis system (Visage 2000; BioImage Corp., Ann Arbor, Mich.), and PBP activity for each band was expressed as the percentage (mean ± standard error) of the integrated band intensity at 0 min.

## RESULTS

*E. coli* cells were incubated with neutrophils and periodically analyzed for membrane penicillin binding activity as described in Materials and Methods. Each of the expected PBPs, designated 1 to 7b, was detectable at the beginning of the incubation (Fig. 1, 0 min), although PBPs 5 and 6 were not individually distinguishable. Penicillin binding was progressively lost over the 2-h exposure to PMNs (Fig. 1), although some activity for each of the PBPs remained even at the final time point.

The decline in penicillin binding was measured by densitometry as described in Materials and Methods. Rates of PMN-mediated inactivation among PBPs varied only slightly (Fig. 2). Binding activity of PBP2 declined most rapidly, with 60% loss of activity after 15 min of exposure, while PBPs 1 and 7 lost only 20% of their activity at the same time point. All of the PBPs had lost >80% of their activity after 120 min of exposure to PMNs. The PMN microbicidal effect on *E. coli* is illustrated at the bottom of Fig. 2. With the exception of PBP2, loss of penicillin binding activity correlated well with loss of viability ($r$ = 0.90 for PBP1, 0.59 for PBP2, 0.74 for PBP3, 0.87 for PBP4, 0.93 for PBPs 5/6, and 0.75 for PBP7), with the best correlations seen for PBPs 1 and 5/6.

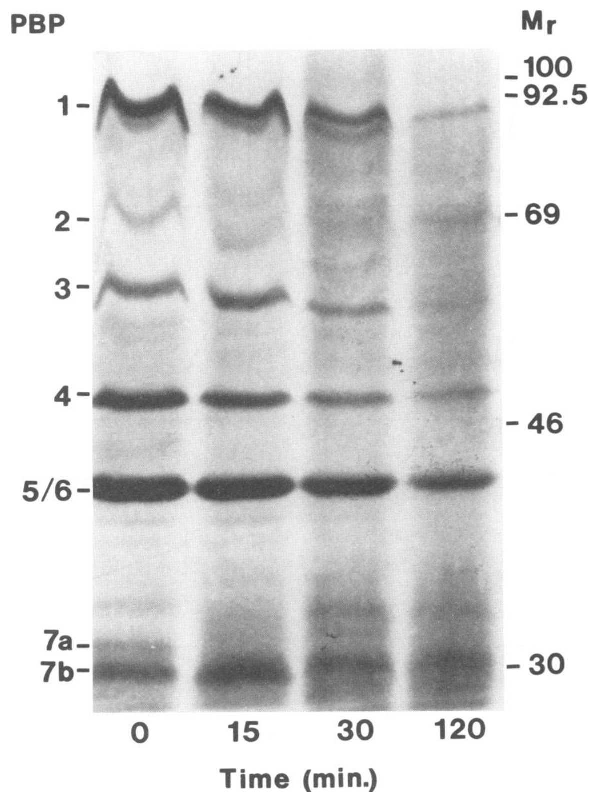


FIG. 3. Effect of azide-treated neutrophils on *E. coli* PBPs. *E. coli* cells were exposed to PMNs, 10% serum, and 5 mM sodium azide, and PBPs were determined as for Fig. 1. Fluorograph is representative of two experiments.

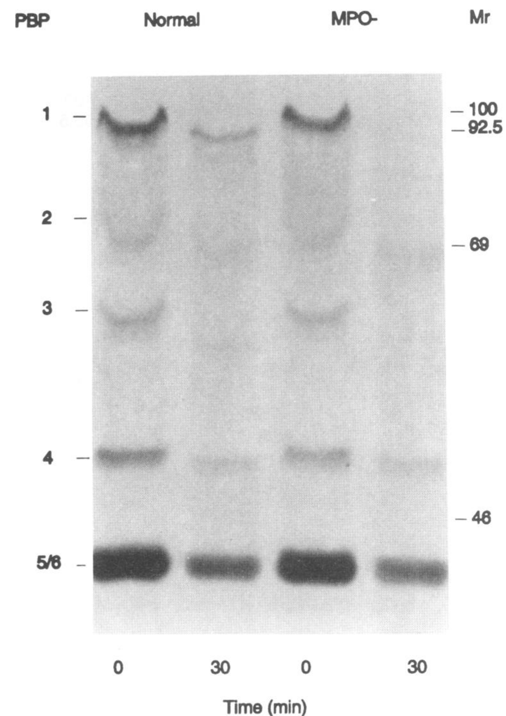


FIG. 4. Effect of MPO-deficient neutrophils on *E. coli* PBPs. *E. coli* cells were exposed to normal or MPO-deficient PMNs, and at 0 and 30 min, PBPs were determined as for Fig. 1.

To explore the contribution of MPO to the loss of penicillin binding activity induced by intact PMNs, we added sodium azide to the PMN-*E. coli* killing mixture to inhibit the activity of MPO (10). Despite a modest decline in microbicidal activity due to azide (41% killing after 30 min versus 66% with normal PMNs), PBP inactivation by azide-inhibited PMNs (Fig. 3) was not appreciably different than that seen with normal PMNs (Fig. 1). However, the interpretation of these results is complicated by the potential for azide to increase $H_2O_2$ levels via inhibition of microbial catalase, which may result in enhanced killing of $H_2O_2$-sensitive organisms (7). Therefore, we examined PBP inactivation with PMNs obtained from a patient with a complete deficiency of MPO (Fig. 4). Although there were only enough PMNs available to test one time point (30 min), there did not appear to be any difference in loss of PBP activity due to MPO-deficient PMNs or normal PMNs.

To investigate the contribution of combined oxygen-dependent microbicidal systems to *E. coli* PBP inactivation, we exposed *E. coli* to PMNs from a patient with CGD, in which there is almost complete loss of respiratory burst activity. Once again, there was no demonstrable difference in PBP inactivation when CGD PMNs were compared with normal PMNs (Fig. 5).

## DISCUSSION

Neutrophils have a diverse array of antimicrobial systems available to combat invading microorganisms. Our data indicate that human PMNs inhibit *E. coli* penicillin binding activity and do so in a manner potentially contributing to microbicidal activity. The loss of penicillin binding strongly suggests enzymatic inactivation of the PBPs, although in rare instances the two processes can be dissociated (19). We

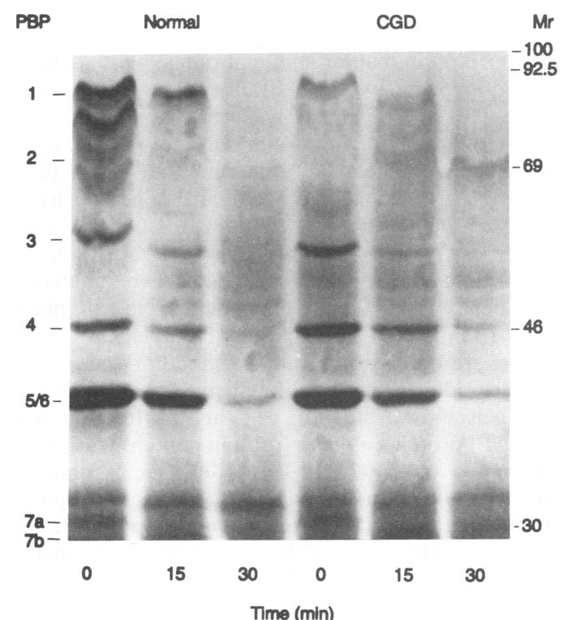


FIG. 5. Effect of CGD neutrophils on *E. coli* PBPs. *E. coli* cells were exposed to normal or CGD PMNs, and at 0, 15, and 30 min, PBPs were determined as for Fig. 1.

found good correlations between loss of PBP activity and microbial viability for almost all of the individual PBPs, with the best correlations for PBPs 1 and 5/6. In *E. coli*, each of the high-molecular-weight PBPs (1, 2, and 3) has been considered essential to bacterial survival, and inhibition of PBP1 by antibiotics results in cell lysis (20). The good correlation between PBP1 inactivation and bacterial death in this study is consistent with the critical nature of PBP1 activity. In the present study, activity of PBPs 2 and 3 declined in a similar manner. As seen in Fig. 2, at 15 min, PBP2 activity had declined to (36 ± 20)% of initial activity, which was not significantly different from the (54 ± 10)% with PBP3. This was unlike our results with the cell-free MPO system, in which there was a large difference between rates of inactivation of PBPs 2 and 3. While PBP3 was particularly sensitive to inactivation in the cell-free MPO system, there was a much more balanced inactivation of all PBPs in response to PMNs, in which a variety of antimicrobial systems are in play. It is unlikely that the loss of penicillin binding seen here with the PMN microbicidal system is merely secondary to death of *E. coli*, since we have previously shown that PBP inactivation is not a simple consequence of microbial cell death with the cell-free systems containing gentamicin or xanthine oxidase-acetaldehyde (13).

Although we have previously observed that MPO in a cell-free system inactivates microbial PBPs, it appears that intact PMNs have other systems which can also act at the same target site. Azide-inhibited PMNs and MPO-deficient PMNs were not appreciably different from normal PMNs in their ability to inhibit *E. coli* PBP activity, indicating that an active MPO system was not necessary for this effect. In addition, since CGD PMNs, lacking a respiratory burst, showed no difference from normal PMNs, nonoxidative mechanisms appear to contribute substantially. One potential nonoxidative mechanism that could be responsible is cathepsin G, which has previously been shown to bind to PBP2 of *Neisseria gonorrhoeae* (16). Lysozyme, another non-oxygen-dependent PMN component, is active in degrading peptidoglycan and could also participate in this process. While nonoxidative PMN microbicidal systems are potent against *E. coli*, the ability of nonoxidative systems to inactivate PBPs and promote killing might be different for other bacteria. For example, *Staphylococcus aureus*, which is primarily killed by oxidative antimicrobial mechanisms, is a prominent pathogen in CGD patients.

In summary, human neutrophils inhibit the penicillin binding activity of critical *E. coli* PBPs at rates similar to those for the loss of microbial viability. While MPO-derived oxidants are active in this process in a cell-free system, whole PMNs also use non-MPO-dependent and, indeed, non-oxygen-dependent, mechanisms in this PBP inhibition. The correlation between the appearance of PBP inactivation and viability loss, together with the known lethality of PBP inactivation from antibiotic studies, suggests that PBP inactivation may be an important bactericidal mechanism used by PMNs.

## ACKNOWLEDGMENTS

This work was supported by grant AI 25606 from the National Institute of Allergy and Infectious Diseases. R.M.R. is a recipient of an Ortho Young Investigator Award of the Infectious Diseases Society of America.

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