# Oxygen-independent Intracellular and Oxygen-dependent Extracellular Killing of *Escherichia coli* S15 by Human Polymorphonuclear Leukocytes

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## Abstract

**Effective killing of bacteria by polymorphonuclear leukocytes (PMN) is generally assumed to require intracellular sequestration and, depending on the bacterial species, can be both $O_2$-dependent or $O_2$-independent. Killing of several strains of *Salmonella typhimurium* and *Escherichia coli* by rabbit PMN does not require $O_2$ and is apparently due to a granule-associated bactericidal/permeability-increasing protein (BPI) present in rabbit and human PMN. In this study we examined the $O_2$ dependence of the killing of *E. coli* (S15) by human PMN. Ingested and noningested *E. coli* were separated by centrifugation after incubation with PMN in room air or under $N_2$. In the presence of heat-treated serum ~50% of *E. coli* (10 bacteria/PMN) were taken up by PMN and rapidly (5–15 min) killed both in room air and under $N_2$. The remaining extracellular bacteria (~50%) were killed during 30–60 min of incubation in room air but not under $N_2$. When uptake of *E. coli* by PMN was increased to ~80% by the use of C6-depleted serum (retaining heat-labile opsonins), bacterial survival under $N_2$ was reduced from 54±7.6% to 13±5.5%. PMN from a patient with chronic granulomatous disease killed PMN-associated but not extracellular *E. coli*. BPI was detected, by indirect immunofluorescence, on the surface of PMN-associated *E. coli* within 5 min of incubation of *E. coli* with PMN both in room air and under $N_2$. In contrast, at no time was BPI detected on the surface of extracellular *E. coli*, indicating that the non-PMN-associated *E. coli* had not been previously ingested. Thus, killing of ingested *E. coli* S15 by human as well as rabbit PMN does not require $O_2$ and appears to be BPI-mediated. However, when ingestion is limited, extracellular bacteria can also be killed but principally by $O_2$-dependent mechanisms.**

## Introduction

Polymorphonuclear leukocytes (PMN) kill bacteria by $O_2$-dependent and $O_2$-independent mechanisms (1, 2). Although substances contributing to both oxidative and nonoxidative modes of killing are released outside the phagocyte as well as into the phagocytic vacuole (3), evidence of substantial extracellular killing of bacteria under normal circumstances is lacking. Hence, effective bacterial killing by PMN is generally presumed to require intracellular sequestration of bacteria (4). We now provide evidence that human PMN do kill extracellular *Escherichia coli* (S15). Extracellular killing of this *E. coli* strain is only effective in the presence of $O_2$, in contrast to killing of ingested *E. coli*, which does not require $O_2$.

## Methods

*PMN.* Heparinized venous blood was obtained after informed consent from healthy volunteers and from a patient with a previously diagnosed, X-linked form of chronic granulomatous disease (CGD)[1] (5). Antibiotic prophylaxis (160 mg of trimethoprim and 800 mg of sulfamethoxazole daily) of the patient was interrupted 60 h before bleeding. PMN were isolated by sedimentation in Ficoll-Paque and dextran T-500 (Pharmacia Fine Chemicals, Piscataway, NJ) (6). Wright stains of blood smears showed that >95% of cells were PMN, ~95% of which were neutrophils and 5% eosinophils. The cells were washed twice with Hanks' balanced salt solution (HBSS; M.A. Bioproducts, Walkersville, MD) before resuspension in HBSS containing 20 mM Tris-HCl, pH 7.5, to a concentration of $4 \times 10^7$ PMN/ml.

*Bacteria. E. coli* S15, a K12 strain ($Leu^-$-$Thr^-$-$Thi^-$-$Lac^-$-$Mal^-$), was kindly provided by Prof. S. Nojima, Faculty of Pharmaceutical Sciences, University of Tokyo.

*Growth and labeling of bacteria.* The bacteria were grown in a triethanolamine-buffered (pH 7.75–7.9) minimal salts medium (7). Stationary-phase overnight cultures were transferred to fresh medium (diluted 1:10) supplemented with either L-amino acids mixture [$^{14}$C(uniform)], 0.5 μCi/ml; >250 Ci/mol; or [2-$^{14}$C]thymidine, 0.5 μCi/ml; 62 Ci/mol (New England Nuclear, Boston, MA) plus cold uridine (2 mM) to prelabeled bacterial proteins or DNA, respectively. After growth to midlogarithmic phase (2.5–4 h at 37°C; 0.5–1 × $10^9$ bacteria/ml, determined by measuring absorbance at 550 nm), the bacteria were harvested by centrifugation at 6,000 *g* for 10 min, resuspended in growth medium without radioactive precursors, and incubated for 30 min at 37°C to chase residual precursors into macromolecules. The bacteria were then pelleted again and resuspended in sterile physiologic saline to 5 × $10^8$/ml. Radiolabeled *E. coli* contained >1,500 cpm/$10^7$ bacteria, >95% of which were precipitable in cold 5% trichloroacetic acid.

*Sera.* Sera collected from many normal human donors were pooled. Heat-treated serum was prepared by incubation of serum at 56°C for 45 min before use. C6-depleted serum was prepared by immunoaffinity chromatography of normal (untreated) serum on Affi-Gel 10 (Bio-Rad Laboratories, Richmond, CA) containing covalently coupled goat (anti-human C6) IgG (Calbiochem-Behring Corp., San Diego, CA), prepared according to the instructions of the manufacturer. Recovery of unbound human serum protein (>95%) was measured by the modified Coomassie blue assay (8). Depletion of C6 was monitored by double immunodif-

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1. *Abbreviations used in this paper:* BPI, bactericidal/permeability-increasing protein; CGD, chronic granulomatous disease; PBSA, phosphate-buffered saline containing 0.2% bovine serum albumin.

fusion of unbound human serum protein against goat (anti-human C6) antibody (9).

Serum was collected from a New Zealand White rabbit before and after immunization with purified human bactericidal/permeability-increasing protein (BPI) (10) to provide preimmune and immune (anti-BPI) sera. To deplete immune sera of anti-BPI antibodies, antiserum was incubated with purified BPI (2 h at room temperature, then 24 h at 4°C) and spun (10,000 *g* for 10 min) to remove immune complexes. Antibody titer was determined by double immunodiffusion of serum against BPI (9). Anti-BPI serum produced a single-fused precipitin line against purified BPI and crude PMN extracts.

*Incubation mixtures.* Typical incubation mixtures contained 0.25 ml of HBSS/20 mM Tris-HCl (pH 7.5)±PMN ($10^7$), 0.05 ml of heat-treated pooled human serum (or saline), and 0.20 ml of *E. coli* ($10^8$) in 10-ml siliconized Vacutainer tubes (no additive; Beckton, Dickinson & Co., Rutherford, NJ).

*Incubation conditions: $O_2$ depletion.* Before mixing PMN and *E. coli*, individual samples containing PMN and serum and stock bacterial suspensions were equilibrated at room temperature in room air or under $N_2$ to create aerobic or $O_2$-depleted conditions. $N_2$ (prepurified; Ohio Medical Products, Madison, WI) flushing was accomplished via 21- (inlet) and 18- (outlet) gauge needles inserted through the rubber stopper that seals these tubes. Flushing was carried out for 20–30 min (longer flushing, up to 60 min, did not alter the results). PMN suspensions were periodically shaken by hand to reduce cell clumping. The bacteria were added to tubes containing PMN via syringe. $N_2$ flushing was continued for 5 min before removing the needles and sealing the tubes. Incubations were carried out in a rotating, (~100 rpm) waterbath at 37°C and stopped by placing the tubes in ice and diluting the suspensions fourfold with ice-cold sterile saline containing sodium heparin (50 U/ml) to block further phagocytosis and killing (11). The bacteria did not multiply during 60 min of incubation in the absence of PMN (100±4.3% and 95±2.5% of 0 min viability in the absence and presence of serum, respectively). $N_2$-flushed PMN generated almost no respiratory burst (<3% of normal), measured either by hexose monophosphate shunt activity or by $O_2^-$ production (12).

*Measurement of uptake of E. coli by PMN.* After cooling and dilution of the cell suspensions, 1 ml was transferred by syringe to a second $N_2$-flushed tube for measurement of bacterial viability in whole cell suspensions (see below). 1 ml of 1.0 M sucrose was injected beneath the remaining 1 ml of cell suspension and free and PMN-associated *E. coli* were separated by centrifugation at 100 *g* for 10 min in a swinging bucket rotor. The PMN sedimented through the sucrose layer. The supernatant (~2 ml) was carefully removed (<100 μl remained) and the PMN pellet was resuspended in saline-heparin to the volume of the supernatant fraction. Bacterial radioactivity in the resuspended PMN pellet ("PMN associated") and in the supernatant fraction ("extracellular") were measured by conventional liquid scintillation counting procedures (11). Supernatant *E. coli* were also enumerated by light microscopy using a Petroff–Hauser bacterial counting chamber. Bacterial uptake by PMN was measured as: (*a*) percentage of total recovered bacterial radioactivity present in the resuspended PMN pellet; and (*b*) percent reduction in the number of *E. coli* in the supernatant. The distribution of bacterial radioactivity was comparable using $^{14}$C-amino acid or [$^{14}$C]thymidine-labeled *E. coli* and the supernatant radioactivity corresponded closely (±10%) to the number of *E. coli* enumerated under the microscope. Total recovery of bacterial radioactivity was 96±3.1%; 96±0.7% of *E. coli* were recovered in the supernatant fraction in the absence of PMN ($n$ = 8).

To determine the properties of uningested bacteria and of the cell-free incubation mixture, cell suspensions were centrifuged at 100 *g* for 10 min.

*Measurement of bacterial viability.* To measure bacterial viability (i.e., colony-forming ability), samples were serially diluted in sterile saline and plated on nutrient agar. Samples of whole cell suspensions were taken both before and after sonication of the suspensions (40 W for 15 s at 0–4°C; Sonifier Cell Disrupter, Heat Systems-Ultrasonics, Inc., Farmingdale, NY) to determine extracellular and total (extra- and intracellular) bacterial viability, respectively. Sonication under the indicated conditions does not affect bacterial viability, but lyses PMN, releasing intracellular bacteria. The viability of PMN-associated and of extracellular *E. coli* was measured only after sonication. The number of colony-forming units on the plates was determined after incubation at 37°C overnight.

*Immunofluorescence.* Samples (5–10 μl) of sonicated whole cell suspensions, resuspended PMN pellets and supernatants, and *E. coli* preincubated±BPI were dried on precleaned microscope slides and fixed (1 min at 4°C) in buffered formalin acetone (13). The fixed smears were soaked for 15 min in Dulbecco's phosphate-buffered saline (PBS), and rinsed with PBS supplemented with 0.2% bovine serum albumin (PBSA). Smears were overlaid for 10 min in a moist chamber at room temperature with 10% heat-treated pooled human serum, to occupy nonspecific binding sites, and rinsed with PBSA. Next, smears were overlaid with either 0.5% rabbit anti-BPI, preimmune, or depleted serum for 30 min, washed three times for 30 min with PBS, rinsed with PBSA, and finally overlaid with 1% rhodamine-labeled swine (anti-rabbit Ig) Ig (Accurate Chemical & Scientific Corp., Westbury, NY) for 30 min. All sera were diluted in PBSA. Excess rhodamine-labeled Ig was removed in three washes (30 min) with PBS and the smears were covered with glycerol:PBS (9:1, vol/vol) and a coverslip and examined under oil using a Leitz Orthoplan fluorescent microscope (E. Leitz, Inc., Rockleigh, NY). Immunofluorescent staining of *E. coli* pretreated with purified BPI was not reduced either by sonication or by subsequent addition of heparin±heat-treated human serum. Heparin prevents attachment of unbound BPI to *E. coli* (14); *E. coli* exposed to BPI in the presence of heparin (50 U/ml) were not stained. Photographs were taken with Kodak Tri-X pan (ASA 400) film at approximately × 1,000 magnification. The micrographs shown in Fig. 2 were further enlarged 3–4× during photography.

*Radioimmunoassay. E. coli* preincubated±BPI or recovered in supernatants of PMN suspensions were sedimented by centrifugation at 10,000 *g* for 10 min to remove unbound protein. The bacteria were resuspended in isotonic saline containing 1% Triton X-100, 1% sodium deoxycholate and 1 mM phenylmethyl sulfonyl fluoride ($2 \times 10^8$/ml), sonicated (as above), and incubated for 60 min at room temperature. Aliquots (20 μl) of the bacterial extracts were dried on 1.3 cm$^2$ strips of nitrocellulose paper (Bio-Rad Laboratories). The strips were then soaked in Tris (10 mM; pH 7.5)-buffered saline, containing 3% bovine serum albumin to saturate protein-binding sites before incubation with 1% rabbit (anti-BPI or preimmune) serum and, after several washes with Tris-buffered saline, with $^{125}$I-goat (anti-rabbit IgG) F(ab)$_2$ ($2 \times 10^5$ cpm) (New England Nuclear) and unlabeled goat (anti-rabbit IgG) IgG (10 μg/1.5 ml) (Miles Scientific, Naperville, IL). All antibody preparations were diluted in Tris-buffered saline supplemented with 3% albumin, 0.5% nonidet P-40, 5 mM EDTA, and 0.05% $NaN_3$. The nitrocellulose strips were again washed with Tris-buffered saline before counting in a gamma counter. Extracts of *E. coli*, killed by BPI, bound $4–5 \times 10^3$ cpm (above background) when treated with anti-BPI rabbit serum and <30 cpm when treated with preimmune rabbit serum.

## Results

*Reduced killing of E. coli S15 by human PMN during $O_2$ deprivation.* Killing of *Salmonella typhimurium* and *E. coli* by rabbit PMN is equally effective in room air and under $N_2$ (12; Elsbach, P., L. Kao, M. Victor, and J. Weiss, unpublished observations). However, killing of *E. coli* S15 by human PMN was markedly reduced under $N_2$ (Table I). Human PMN, unlike rabbit PMN (12, 15), are unable to kill rough strains of gram-negative bacteria such as *E. coli* S15 in the absence of serum (data not shown). In the presence of 10% heat-treated

*Table I. Killing of E. coli S15 by Human PMN Under Room Air and $N_2$*

| Incubation time | Bacterial viability | | | |
|---|---|---|---|---|
| | Room air | | Nitrogen | |
| | −Sonic. | +Sonic. | −Sonic. | +Sonic. |
| *min* | *% of E. coli incubated without PMN* | | | |
| 15 | NT‡ | 49±3.2 | NT | 63 |
| 30 | 22±4.2 | 23±3.4 | 61±8.9 | 62±5.8 |
| 60 | 8±4.7 | 10±2.1 | 44±7.7 | 47±4.3 |

*E. coli* S15±PMN (10 *E. coli*/PMN) were incubated in the standard incubation mixture (containing 10% heat-treated serum) at 37°C for the indicated time under room air or $N_2$. Bacterial viability was measured before and after sonication of cell suspensions as described in Methods. The values shown represent viability of *E. coli* incubated with PMN expressed as percent of viability of *E. coli* incubated without PMN and are the mean (±SEM, where indicated) of results obtained in from 2 to 13 experiments. Viability of sonicated *E. coli* suspensions (no PMN) is 99±5.2% of unsonicated bacterial suspensions. Sonic., sonication; NT, not tested.

serum, the viability of *E. coli* incubated with human PMN in room air decreased progressively, falling to <10% after 60 min (Table I). Under $N_2$, most killing took place within the first 15 min and nearly 50% of the added bacteria remained viable after 60 min. After sonication to release ingested *E. coli*, the number of viable bacteria measured was nearly the same as before sonication indicating that there was no appreciable intracellular survival and, hence, that *E. coli* surviving under $N_2$ were extracellular.

*Evidence of $O_2$-independent intracellular killing and $O_2$-dependent extracellular killing.* To assess more directly the location of live and dead *E. coli* after incubation with PMN, PMN-associated and extracellular radiolabeled *E. coli* were separated by low-speed centrifugation. Maximally, only about half of the added *E. coli* were recovered in the PMN pellet whether incubated in room air or under $N_2$ (Fig. 1); this fraction was not increased by reducing the number of *E. coli* added per PMN (from 10 to 3) or by increasing the amount of serum added (up to 40%). Most uptake occurred within the first 5–15 min and PMN-associated bacteria were efficiently killed in room air as well as under $N_2$ (Fig. 1). After 5 min in room air, >50% of *E. coli* recovered in the PMN pellet were nonviable and nearly all were dead after 30 min. The rate and extent of killing of PMN-associated bacteria were only slightly less under $N_2$ (Fig. 1).

In contrast, the viability of the remaining extracellular *E. coli* differed greatly in room air and under $N_2$ (Fig. 1). Initially (5–15 min), most *E. coli* recovered in the extracellular medium of aerobic and $O_2$-depleted cell suspensions were viable. After longer incubation in room air, nearly all (84±4.5%) recovered extracellular bacteria were nonviable. However, after 60 min of incubation under $N_2$, only a small fraction (21±7.7%) of the extracellular *E. coli* were killed. Thus, reduced killing of *E. coli* S15 under $N_2$ appears to be attributable to increased survival of extracellular but not of intracellular bacteria.

These findings suggest two mechanisms of killing of *E. coli* S15 by human PMN: $O_2$-independent (i.e., $O_2$ is not required)

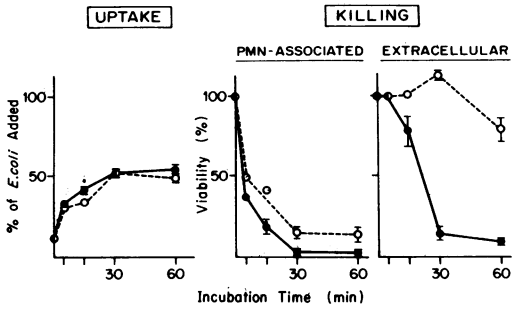


*Figure 1.* Intracellular and extracellular killing of *E. coli* S15 by PMN in room air or $N_2$. *E. coli* and PMN (10 *E. coli*/PMN) were incubated for various periods in the standard incubation mixture (containing 10% heat-treated serum) at 37°C in room air (●) or under $N_2$ (○). PMN-associated and extracellular bacteria were separated by centrifugation (see Methods). Uptake of *E. coli* by PMN and the viability of PMN-associated and extracellular bacteria were measured as described under Methods. Bacterial uptake (measured by radioactive and/or microscopic counting; see Methods) is expressed as the percentage of added *E. coli* which is recovered in the PMN pellet. Bacterial viability is expressed as the percentage of bacteria recovered in each fraction which is viable. The results shown represent the mean (±SEM, where indicated) of from two to seven experiments.

intracellular killing and $O_2$-dependent extracellular killing. This conclusion is supported by results obtained in an experiment with PMN from a patient with CGD whose leukocytes cannot generate toxic $O_2$ metabolites (1). As shown for $O_2$-depleted normal PMN, CGD leukocytes killed nearly all PMN-associated but few extracellular *E. coli* (Table II). Overall killing of *E. coli* S15 by CGD leukocytes was only slightly less than by normal PMN apparently because a greater fraction of the bacteria were taken up by the patient's PMN (76% vs. 55±2.7% (54% in this experiment)) and killed by $O_2$-independent mechanisms. The patients' PMN did not kill *Staphylo-*

*Table II. Normal Intracellular but Defective Extracellular Killing of E. coli S15 by CGD Leukocytes*

| PMN (atmosphere) | Uptake | *Viability of *E. coli* in | | |
|---|---|---|---|---|
| | | Whole suspension | PMN pellet | Supernatant |
| | % | % | % | % |
| Normal (room air) | 55±3 | 10±2 | 4±1 | 14±5 |
| Normal ($N_2$) | 48±3 | 47±4 | 14±4 | 79±8 |
| CGD (room air) | 76 | 17 | 3 | 71 |

*E. coli* S15 were incubated for 60 min at 37°C in the standard incubation mixture (containing 10% heat-treated serum)±PMN under room air or $N_2$. Bacterial uptake and viability were measured as described under Methods. The effects of CGD leukocytes that are shown represent the results obtained in a single experiment. The results (mean±SEM) shown for normal PMN (incubated either in room air or under $N_2$) are shown for comparison and represent the data shown in Table I and Fig. 1 to which were added those of two controls included in this experiment.

* The numbers shown represent the percent of bacteria recovered after 60 min of incubation in whole suspension, PMN pellet, or supernatant (determined as described in Methods) that were viable.

*coccus aureus,* a bacterial species that is not effectively killed by $O_2$-independent bactericidal mechanisms (1, 12). This indicates that omission of antibiotics for 60 h before blood was withdrawn from the patient eliminates the effect of the antibiotics on the bactericidal potential of the PMN in vitro (16).

*Granule-associated BPI is bound to the surface of PMN-associated, but not of extracellular, E. coli.* To verify that extracellular *E. coli* represent noningested rather than previously ingested and extruded bacteria, we examined the bacteria after incubation with PMN to determine whether BPI, a granule-associated protein, had attached to *E. coli.* BPI, in crude PMN fractions as well as in purified form, has a high affinity for *E. coli* (12, 17) and rapidly binds to the bacterial surface (18). However, 10% serum (added to suspensions with intact PMN) inhibits binding of BPI to *E. coli* (unpublished observations). Thus, transfer of BPI from PMN granules to the surface of *E. coli* S15 can only occur inside, not outside the PMN.

BPI binding to *E. coli* was readily seen by indirect immunofluorescence (Fig. 2). *E. coli* killed by either crude (granule-rich) cell-free PMN fractions or by purified BPI show bright fluorescent staining after fixation and treatment with rabbit anti-BPI serum and rhodamine-labeled swine (anti-rabbit IgG) antibodies (Fig. 2, top left). No bacterial staining was seen when BPI-killed *E. coli* were treated with preimmune serum (Fig. 2, bottom left panel) or immune serum depleted of anti-BPI antibodies, nor when either immune (Fig. 2, top right), preimmune (Fig. 2, bottom right) or depleted antisera were used to treat *E. coli* that had not been exposed to BPI. Thus bacterial fluorescence is specifically due to the reaction of anti-BPI antibodies with BPI bound to *E. coli.*

*E. coli* recovered in the PMN pellet after aerobic or anaerobic incubation with intact PMN were also brightly fluorescent when treated with anti-BPI serum (Fig. 3, top center) but not when treated with preimmune serum (Fig. 3, bottom center). After 5 min of incubation, most PMN-associated bacteria were stained; after 15 min, essentially all *E. coli* were fluorescent. In contrast, no extracellular *E. coli* were stained at any time (up to 60 min) (Fig. 3, top right) nor was any bound BPI detectable on extracellular *E. coli* by radioimmunoassay (sensitivity limit: <3% of lethal BPI dose). Whole suspensions of *E. coli* and PMN sonicated without prior incubation also showed little or no bacterial fluorescence (Fig. 3; top left). Therefore, the fluorescence of PMN-associated *E. coli* reflects the rapid binding of BPI to *E. coli,* which occurred within the intact neutrophil and not during the disruption of PMN by sonication. Hence the absence of detectable BPI on extracellular *E. coli* confirms that these bacteria were not ingested at any time and, in room air, were killed extracellularly.

*Effects of serum on uptake and killing by PMN.* When viable extracellular *E. coli,* recovered from $O_2$-depleted PMN suspensions, were incubated with a fresh population of PMN, roughly 50% of the bacteria were again ingested indicating that the noningested bacteria were not intrinsically resistant to phagocytosis. To determine whether limited ingestion was due to weak opsonizing activity of heat-treated serum, we prepared C6-depleted serum, which retains the opsonic but not the bactericidal activity of the heat-labile complement system (19). Indeed, when heat-treated serum was replaced by C6-depleted serum, bacterial uptake by PMN increased to nearly 80% (Table III). Again, nearly all PMN-associated *E. coli* were killed in $O_2$-depleted as well as normally aerated cell suspensions. Consequently, overall bacterial killing under $N_2$ was substantially greater when C6-depleted, rather than heat-treated, serum was used and was only slightly less than bacterial killing in room air (Table III). The somewhat greater killing under $N_2$ of extracellular bacteria, when C6-depleted serum was used,

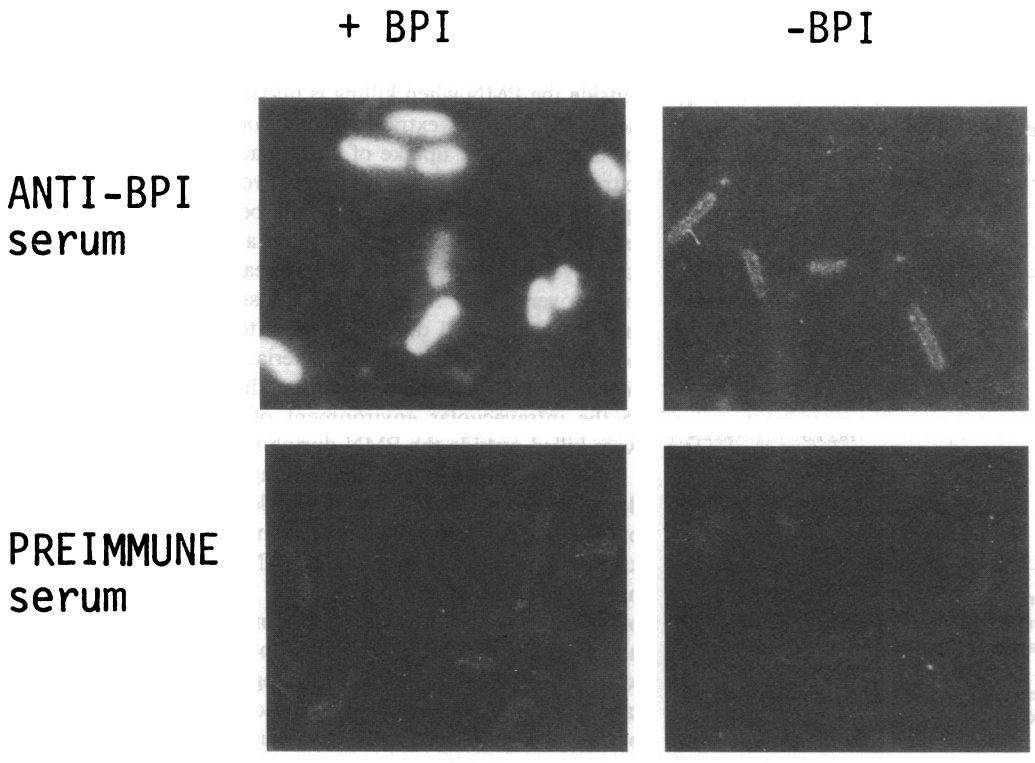


*Figure 2.* Detection of BPI binding to *E. coli* S15 by indirect immunofluorescence. *E. coli* ($3 \times 10^7$/ml) were incubated for 5 min in the presence (*left*) or absence (*right*) of BPI (3 μg/ml). Indirect immunofluorescent staining of *E. coli* was carried out as described under Methods. Bacteria shown in upper and lower panels were treated with rabbit anti-BPI and preimmune sera, respectively. Viability of BPI-treated bacteria was <5% of untreated *E. coli.*

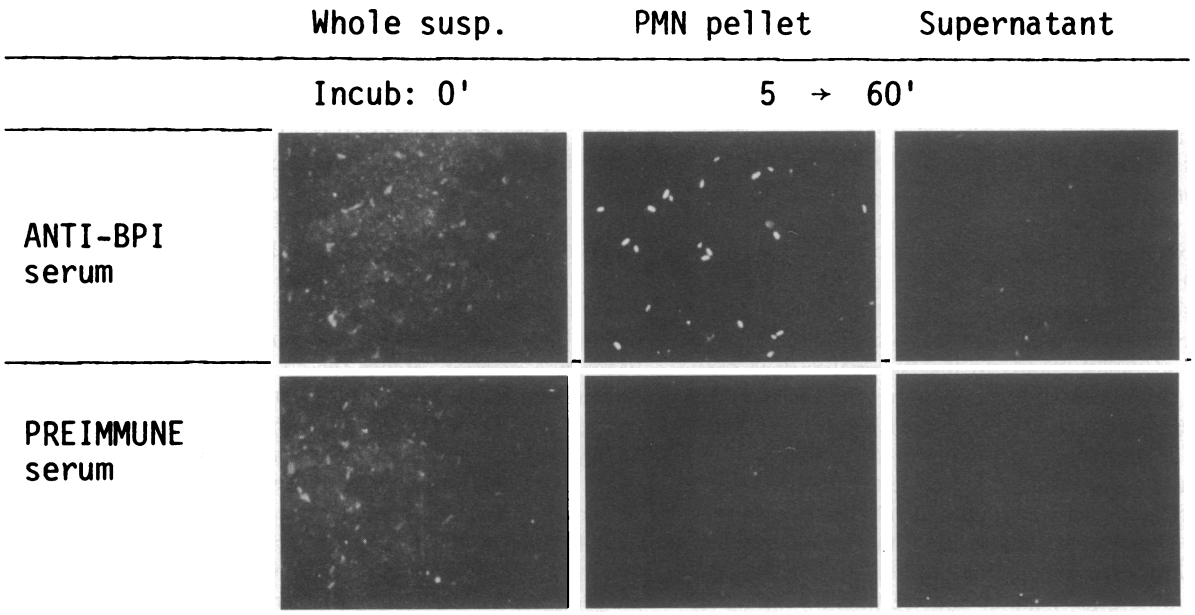


*Figure 3.* Immunofluorescent staining of *E. coli* S15 incubated with intact PMN. Indirect immunofluorescent staining of whole suspensions (susp.) of PMN and *E. coli* (mixed at 4°C; *left*), resuspended PMN pellets (*center*), and extra-PMN supernatants (*right*) were carried out as described in Methods after sonication of cells to disperse the bacteria. Bacteria shown in upper and lower panels were treated with rabbit anti-BPI and preimmune sera, respectively. The pattern of staining of PMN-associated and of extracellular *E. coli* after 5, 15 (shown), 30, or 60 min incubation of *E. coli* with intact PMN was similar.

might reflect the release by PMN of weak $O_2$-independent bactericidal activity evident only when small numbers of uningested *E. coli* remain.

## Discussion

Extracellular cytotoxicity of PMN has been demonstrated toward a wide variety of cells including both large prokaryotes and eukaryotes (3). Extracellular killing of bacteria has also been shown, but only under artificial conditions, e.g., by adding cytochalasin D to block phagocytosis (20) or phorbol myristate acetate to stimulate oxidative metabolism and degranulation by PMN incubated with nonopsonized (noningested) bacteria (21).

*Table III. Effect of Heat-treated and C6-depleted Serum on Uptake and Killing of E. coli S15 by Human PMN in Room Air and Nitrogen*

| Serum | Effect on *E. coli* | | Room air | Nitrogen |
|---|---|---|---|---|
| Heat-treated | Uptake (%) | | 57±2.2 | 51±2.4 |
| | Viability (%) in: | PMN pellet | 0.2±0.2 | 7.4±3.3 |
| | | Supernatant | 22±7.1 | 102±14 |
| | | Whole suspension | 9.4±2.9 | 54±7.6 |
| C6-depleted | Uptake (%) | | 78±1.3 | 79±2.4 |
| | Viability (%) in: | PMN pellet | 0.3±0.3 | 1.8±0.7 |
| | | Supernatant | 20±8.7 | 48±11 |
| | | Whole suspension | 5.7±2.3 | 13±5.5 |

*E. coli* were incubated in room air or $N_2$ for 30 min at 37°C in the standard incubation mixture containing 10% heat-treated or C6-depleted serum±PMN. Bacterial uptake and viability were measured as described under Methods and in the legends to Fig. 1 and Table II. The results shown represent the mean±SEM of five independent determinations. The viability of *E. coli* treated with 10% C6-depleted serum (−PMN) was 98.2±7.3% ($n$ = 6) of bacteria incubated alone.

This study demonstrates substantial extracellular killing of *E. coli* by phagocytizing human PMN, using opsonin-deficient heat-treated serum to permit only partial uptake of bacteria. Although ingested *E. coli* are killed more rapidly, nearly equal numbers of dead bacteria are located within and outside the PMN when killing is maximal. The gradual reduction in viability of extracellular *E. coli* could conceivably be explained by slow uptake of viable bacteria concomitant with extrusion of dead *E. coli*. However, two findings strongly argue against this possibility. First, the number and fraction of PMN-associated *E. coli* killed under room air and $N_2$ is almost the same, yet appreciable numbers of dead extracellular bacteria are recovered only from aerobic cell suspensions. Second, the granule protein BPI rapidly attached to ingested *E. coli* but is not detected on extracellular bacteria. It thus appears that *E. coli* recovered in the extracellular medium were never exposed to the intravacuolar environment of the PMN and, hence, were killed outside the PMN during incubation in room air.

The defect in extracellular killing exhibited by both $O_2$-depleted normal PMN and CGD leukocytes indicates that oxidative mechanisms play an essential role in extracellular killing of *E. coli* S15 by human PMN. Extracellular killing is not seen in the absence of serum, presumably because the oxidative metabolism of the PMN is not adequately stimulated under these conditions. Which, of the many potentially cytotoxic $O_2$ metabolites generated during the respiratory burst, is (are) primarily responsible for extracellular killing of *E. coli* by the human PMN is still uncertain. Passo and Weiss (21)

have shown that oxidative killing of extracellular *E. coli* by phorbol myristate acetate-stimulated PMN is mediated principally by the myeloperoxidase-$H_2O_2$-$Cl^-$ system, and we find, in preliminary experiments, that azide and cyanide, inhibitors of myeloperoxidase, reduce killing of *E. coli* by PMN in room air to roughly the same levels observed under $N_2$. In both studies, the rate of extracellular killing is slow (relative to intracellular killing), possibly reflecting the action of long-lived, slowly reactive oxidants such as hydrophilic *N*-chloramines that accumulate in the extracellular medium (21–24) and can be bactericidal (22, 23). However, in agreement with Passo and Weiss, we do not find evidence of bactericidal activity in the supernatant of suspensions of PMN and *E. coli* (tested toward freshly added *E. coli*) suggesting that slow killing may reflect relatively slow accumulation of short-lived oxidants on the bacterial surface (21).

Although toxic $O_2$ metabolites may also contribute to killing of PMN-associated *E. coli*, the virtually normal killing of these bacteria by $O_2$-depleted normal PMN and by CGD leukocytes indicates that $O_2$-independent mechanisms alone are sufficient to kill ingested *E. coli*. The slight increase in the number of viable *E. coli* recovered in the PMN pellet after incubation under $N_2$ may be due at least in part to trapping of a small fraction (10%) of live extracellular bacteria. The fact that increased bacterial ingestion (replacing heat-treated serum with C6-depleted serum) results in a corresponding reduction in bacterial survival under $N_2$ supports this view and underscores the effectiveness of $O_2$-independent intracellular killing of this strain of *E. coli*. Oxygen-independent killing of several strains of *E. coli, S. typhimurium,* and certain other species of gram-negative bacteria by rabbit and human PMN has previously been shown (12, 25–27). Both species of PMN possess multiple nonoxidative bactericidal systems (2, 28). In both rabbit and human PMN, the principal $O_2$-independent bactericidal agent toward these gram-negative bacteria appears to be BPI, judging from the potency of BPI (in cell-free PMN fractions) and the similarity of the lesions initially inflicted by isolated BPI and by intact PMN (10, 12, 29). The demonstration in this study that BPI rapidly binds to ingested *E. coli* satisfies a primary requisite for its action in the intact neutrophil (17, 18).

The absence of detectable BPI on noningested bacteria may explain the $O_2$ dependence of extracellular killing of *E. coli*. This compartmentalization of $O_2$-independent killing and the use of opsonin-deficient conditions have made this a particularly favorable setting for the demonstration of concomitant intra- and extracellular killing as well as of a previously unrecognized defect in extracellular killing by CGD leukocytes. Although heat-treated serum is a laboratory artifact, many situations occur in vivo in which (heat-labile) opsonins are deficient (19). The ability of PMN to generate toxic $O_2$ metabolites against extracellular microorganisms provides an effective ("back-up") bactericidal mechanism which may be important under conditions when intracellular sequestration of bacteria is incomplete.

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