Targeting Lipopolysaccharides by the Nontoxic Polymyxin B Nonapeptide Sensitizes Resistant *Escherichia coli* to the Bactericidal Effect of Human Neutrophils

Frank Rose, Kay U. Heuer, Ulf Sibelius,
Sabine Hombach-Klonisch, Ladislau Kiss,
Werner Seeger, and Friedrich Grimminger

Department of Internal Medicine, Justus-Liebig-University,
Giessen, Germany

The nonapeptide of polymyxin B (PMBN) has been reported to sensitize various pathogenic gram-negative bacteria to the direct bactericidal effect of human serum. To investigate the impact of PMBN on human neutrophil-effected killing of the serum- and phagocytosis-resistant *Escherichia coli* strains C14 and O111, serum was coapplied with PMBN or with neutrophils, but this did not result in decreased numbers of viable bacteria. In contrast, the most potent bacterial killing occurred in the presence of neutrophils plus serum components plus PMBN. The effect of this on *E. coli* C14 was the appearance of inositol phosphates, diacylglycerol, respiratory burst, elastase liberation, and generation of lipid mediators (leukotriene B4, 5-HETE, and platelet-activating factor). Strong neutrophil activation required early, but not late, complement components and was blocked by inhibition of phagocytosis with cytochalasin D. PMBN seems to cause dramatic support of natural host defense by complement-dependent sensitization of *E. coli* to the bactericidal efficacy of human neutrophils.

*Escherichia coli* is a clinically relevant human pathogen. It causes severe infection, sepsis, and sepsis-related organ injury, which is an important cause of death in critically ill patients [1, 2]. Its virulence is dependent on several factors. First, O-antigenic lipopolysaccharides (LPSs) of the outer membrane, liberated upon bacterial breakdown, are potent inductors of host inflammatory events, linked to the clinical appearance of sepsis and septic shock [3]. Second, ~50% of *E. coli* isolates causing extraintestinal infections in humans secrete α-hemolysin, a proteinaceous, pore-forming, microbial α-toxin, which attacks immunocompetent and endothelial cells and triggers cell death and inflammatory processes [4–7]. Third, many strains of *E. coli* are resistant to the lytic action of the complement cascade and to phagocytosis by leukocytes. Such resistance of bacteremic *E. coli* has been linked to the coating of the outer membrane with long hydrophilic polysaccharide structures, as encountered in the smooth strains, and to the presence of the low immunogenic K1 capsule [8–11]. Activation of the complement cascade is thereby suppressed, with the opsonic C3b and the lytic terminal complex occurring less often. In addition, the access of this lytic complement complex to the hydrophobic domains of the bacterial membrane, with subsequent membrane insertion and bacterial lysis, is hampered.

Recruitment of polymorphonuclear neutrophils to an infectious focus is a major line of defense against bacterial invasion. By phagocytosis of the bacteria and their intracellular “coordinated” breakdown, liberation of bacterial products such as LPS is avoided. The phagocytosis process is intimately coupled to intracellular signaling events, giving rise to mediator generation for destruction of bacteria (oxygen radicals and proteases) and for autocrine neutrophil activation and attraction of additional leukocytes to the microbial focus (lipid mediators such as leukotriene B4 [LTB4] and platelet-activating factor (PAF) [12–15]).

Polymyxin B nonapeptide (PMBN) is a deacylated derivative of the natural antibiotic polymyxin B lacking the fatty acid part of the parent compound. PMBN thereby lost the bactericidal activity of polymyxin B and is noticeably far less toxic—or even nontoxic [11]. PMBN does, however, still possess endotoxin-binding activity via interacting with the anionic groups of LPSs [11, 16, 17], although the deacylated derivative is also less active in its anti-endotoxin activity than that of its parent compound [18]. By such interaction at the surface of viable gram-negative bacteria, this nonapeptide unshields deep structures, including the hydrophobic outer membrane domains, thus facilitating the insertion of the membrane attack complex of the complement system into the bacterial membrane [19, 20]. It thus sensitizes smooth and K1-encapsulated strains to the bactericidal action of serum. In addition, the outer membrane-disorganizing effect of PMBN was described to enhance the
permeability of the bacterial membrane for hydrophobic antibiotics [21–23].

We now extended these previous studies by investigating the effect of PMBN on phagocytosis and killing of the serum-resistant smooth E. coli strains C14 and O111 by human neutrophils in the presence of serum. In contrast to the K1-encapsulated E. coli C14, the viability of E. coli O111 is largely attributed to the presence of lipid A–bound heteropolysaccharides on the cell surface. These polysaccharides inhibit bacterial agglutination and are major acceptor sites for C3 deposition without endotoxin activity [24, 25]. We corroborated the finding of PMBN-effected E. coli sensitization to serum, as assessed in the absence of neutrophils. The most striking observation, however, was a dramatically enhanced bacterial killing upon coapplication of PMBN and leukocytes. Evidence is presented that this hitherto unappreciated PMBN effect proceeds via activation of early complement components, markedly increased bacterial phagocytosis and related neutrophil signaling events, and mediator generation. Such improvement of human neutrophil defense capabilities, effected by a nontoxic agent intercalating the outer bacterial LPS structures, might prove to be a new strategy for antimicrobial therapy.

Materials and Methods

LTB4 and 5-HETE were obtained from Biomol (Hamburg, Germany). All leukotrienes were checked for purity and quantified by spectrophotometry before use, as described elsewhere [26]. Chromatographic supplies included silica gel 5-μm column packing (Shandon, Astmoor, UK), Chromabond C-18sec cartridges (Macherey Nagel, DuÈren, Germany), and high-performance liquid chromatography (HPLC)-grade solvents, distilled in glass (Fluka KG, Neuss, Germany). The lactate-dehydrogenase assay and PMBN were purchased from Boehringer (Mannheim, Germany). The lactate-dehydrogenase assay and PMBN were purchased from Boehringer (Mannheim, Germany). [3H]myo-inositol, [3H]PAF, and [3H]serotonin were obtained from Sigma (Deisenhofen, Germany). The PAF reagent, 1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine, superoxide biotics were obtained from Gibco (Karlsruhe, Germany). The PAF balanced salt solution (HBSS), PBS, trypticase soy broth, and anticomplementary salt solution (Hepes) were obtained from Gibco (Karlsruhe, Germany). RPMI 1640 medium, HEPES, Hanks’ balanced salt solution (HBSS), PBS, trypticase soy broth, and antibiotics were obtained from Gibco (Karlsruhe, Germany). The PAF 1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine, superoxide dismutase, cytochrome c type IV, zymosan, FMLP, and A23187 were obtained from Sigma (Deisenhofen, Germany). The PAF receptor antagonist WEB 2086 was generously supplied by Boehringer Mannheim (Ingelheim, Germany). The lactate-dehydrogenase assay and PMBN were purchased from Bachinger (Mannheim, Germany). [3H]myo-inositol, [3H]PAF, and [3H]serotonin were obtained from Amersham (Dreieich, Germany). Percoll was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). S-2484, a substrate for neutrophil elastase, was purchased from Kabi-Vitrum (Stockholm, Sweden). All other biochemicals were obtained from Merck (Munich, Germany).

Bacterial strains. The K1 antigen–producing serum- and phagocytosis-resistant E. coli C14 was isolated from a patient with neonatal meningitis [27]. The serum- and phagocytosis-resistant K1-negative E. coli O111 is characterized by lipid A–bound O-antigen heteropolysaccharides that are different from LPSs [24, 25] and was generously supplied by Dr. S. Bhakdi (Mainz, Germany).

Preparation of human granulocytes. Heparinized blood from healthy donors was centrifuged in a discontinuous Percoll gradient [28] to yield a neutrophil fraction of ~97% purity. Prior to experiments, neutrophils were kept in RPMI 1640 medium with 10% fetal calf serum for 30–60 min at 37°C. Immediately before experimental use, cells were washed twice and suspended in HBSS HEPES buffer to obtain neutrophil concentrations of 5 × 105 neutrophils/mL. Cell viability, as assessed by trypan blue exclusion, ranged >96%, and lactate dehydrogenase release was consistently <3%.

Preparation of complement-depleted serum. Lyophilized R8/9-depleted serum (depleted of complement factors 8 and 9) was produced by chromatography of fresh human serum over the respective affinity columns at 4°C, as described in detail elsewhere [29]. As documented in that publication, this serum is able to activate early complement components.

Granulocyte-bacteria cocultures. E. coli C14 and O111 were grown overnight at 37°C in 10 mL of trypticase soy broth (pH 7.4) medium. After overnight culture, 20 μL of the bacterial suspension was added to 50 mL of fresh trypsinase soy broth and incubated at 37°C on a roller to the middle log phase (photometrically assessed at 578 nm). Bacteria were then spun at 3000 g and resuspended in HBSS. To obtain suspensions of varying bacterial densities, photometric assessment at 578 nm was performed, and adjustment to the optical densities (ODs) was undertaken (OD 1.0, >115 × 105 cfu/mL; OD 0.1, >13 × 105 cytox mL; and OD 0.01, >1 × 105 cfu/mL). For granulocyte bacteria cocultures, 100 μL of the bacterial suspension was admixed to 900 μL of HBSS buffer (pH 7.4) containing 5 × 105 neutrophils/mL in the presence of the different agents under investigation. After various times, reactions were stopped by admixing trichloroacetic acid (for inositol phosphates) or acetic acid and methanol (for PAF) or by placement on ice for 15 min, with subsequent spinning at 3000 g for leukotrienes, elastase secretion, and respiratory burst.

Measurement of neutrophil bactericidal capacity. Neutrophil bactericidal capacity was analyzed as described elsewhere [30]. Briefly, neutrophils (5 × 107/mL) were mixed with suspensions of E. coli (ODs adjusted to 1.0, 0.1, and 0.01), PMBN (20 μg/mL), and human serum (10%). The tubes were incubated at 37°C in duplicate, and samples were removed after varying periods of incubation for quantitative viable colony-forming units by the pour plate method. The results are given as percentages of living bacteria.

Measurement of phagocytosis. The rate of neutrophil phagocytosis of E. coli was investigated by labeling nonphagocytosed bacteria with 1H-thymidine in an assay adapted to that of Harvey et al. [31]. E. coli (OD adjusted to 1.0) were incubated with 10% v/v R8/9 serum at 37°C in the presence of 10 μM neutrophils, 2.5 μg/mL PMBN, both neutrophils and PMBN, or none of these (control). After an incubation period of 25 min at 37°C to permit phagocytosis, samples were placed on ice for 2 min to stop the phagocytosis process and labeling of the remaining (that is, unphagocytosed and alive) E. coli was performed with 0.2 μCi 1H-thymidine/mL at 37°C. According to preceding pilot experiments, a 10-min incubation time for optimum thymidine uptake has been chosen for both strains. Next, the entire sample volume was passed through a 2-μm filter to retain the neutrophils. The filter membrane and E. coli in the filtrate were washed separately, and labels on the filter membrane (neutrophil-associated, viable E. coli) and in the filtrate (nonphagocytosed, nonadherent E. coli) were measured by liquid scintillation counting. Separate control experiments demonstrated that the quantity of neutrophil-associated label persist-
serum. All data are given as percentage of baseline values and represent viability was observed. Controls represent sham incubations without the presence of both neutrophils and PMBN, dramatic loss of bacterial growth occurred upon coapplication of PMBN and the Bactericidal Effect of Neutrophils.

Myo-[3H]inositol (50 &micro;Ci/mL) was added, and cells were incubated at 37°C for 2 h in a shaking water bath. At different times after stimulus, application samples were quenched with trichloracetic acid, extracted with diethylether, and processed to separate inositol phosphates on Dowex anion exchange columns. The columns were eluted sequentially with water (for free [3H] inositol); 5 &micro;M formic acid/0.2 M ammonium formate (for [3H]inositol monophosphate); 0.1 M formic acid/0.5 M ammonium formate (for [3H]inositol diphosphate); or 0.1 M formic acid/1.0 M ammonium formate (for [3H]inositol triphosphate), and samples were processed for liquid scintillation counting. Under current assay conditions, cyclic inositol monophosphate decomposes quantitatively to generate [3H]inositol monophosphate. All inositol phosphates are summarized as IPx, where IP indicates [3H]inositol and x, replaced with 1, 2, or 3, indicates mono-, di-, or triphosphate, respectively.

**Diacylglycerol (DAG) assay.** Cells and cell supernatant were extracted according to Bligh and Dyer [33]. The chloroform phase was removed and was kept at −20°C to minimize acyl group migration. Diacylglycerol (DAG) was quantified within 24 h by enzymatic conversion to [32P]phosphatidic acid as described by Preiss et al. [34]. After subsequent neutral lipid extraction, an aliquot of the lipid phase was subjected to thin-layer chromatography on a silica gel 60 F254 plate and developed with chloroform:methanol:acetic acid (65:15:5, v/v/v). Identification of DAG and its separation from labeled ceramide phosphate was ascertained by autoradiography before liquid scintillation counting of the DAG spot. DAG recovery and conversion were ascertained to range consistently >85%. The amounts of sn-1,2-DAG present in the original samples were calculated from the respective phosphatidic acid counts and the specific activity of the adenosine triphosphate batch employed.

**Assessment of eicosanoids.** AA metabolites were extracted from cell supernatants by octadecylsilyl solid-phase extraction, as described elsewhere [26, 35]. Isocratic reversed-phase HPLC of eicosanoids was performed on octadecylsilyl columns (4 × 250 mm, Hypersil (Shandon, Astmoor, England), 5-µm particles) by using, as a mobile phase, a 72:28:0.16 (v/v/v, pH 4.9) mixture of methanol, water, and acetic acid. In addition to the conventional UV detection with a variable 1-wavelength detector, a photodiode array detector (Waters model 990; Waters/Millipore, Eschborn, Germany) was employed, which provided full UV spectra (190–340 nm) of eluting compounds and allowed checking for peak purity and subtraction of possible coeluting material. The actual recovery factors were assessed by addition of 0.2 &micro;g of [2H]LTB4 and [2H]5-HETE to the buffer medium as internal standards in selected experiments. For quantification of LTBs and 5-HETE, correspondence of values calculated from UV absorbancy in 2 chromatographic runs was required (deviation <10%).

**Post-HPLC PAF bioassay.** PAF production in neutrophils was quantified by induction of [3H]serotonin release from prelabeled
Table 1. *Escherichia coli* O111 viability, given as mean ± SE percentage of colony-forming units (cfu) in the absence or presence of polymyxin B nonapeptide (PMBN).

<table>
<thead>
<tr>
<th>Time, min</th>
<th>PMBN and neutrophils</th>
<th>Neutrophils only</th>
<th>PMBN without neutrophils</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>52.4 ± 4^a^</td>
<td>92 ± 3</td>
<td>95 ± 5</td>
<td>100</td>
</tr>
<tr>
<td>60</td>
<td>55 ± 2^a^</td>
<td>136 ± 5^a^</td>
<td>160 ± 15</td>
<td>185 ± 9</td>
</tr>
</tbody>
</table>

NOTE. *Escherichia coli* O111 (OD 1.0) were incubated with serum (10% v/v) for various times in the presence of both neutrophils and PMBN, neutrophils only (1 × 10^9/mL), PMBN only (20 μg/mL), or none of these (control). Progressive bacterial growth in Hanks’ balanced salt solution buffer in the presence of serum alone occurred to cfu (% of baseline) within 20 min and was set at 100% (control). Upon coagulation of both neutrophils and PMBN, a dramatic loss of bacterial viability was observed within 20 min. In the presence of neutrophils without PMBN, the bacterial growth was only slightly reduced after 60 min. PMBN without neutrophils had no significant effect. Values given are mean ± SE of 4 independent experiments.

*Significantly different from controls (P < 0.05).

Table 2. Postlabeling of *Escherichia coli* C14 undergoing incubation with neutrophils and filter passage in the absence or presence of polymyxin B nonapeptide (PMBN).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Filter membrane counts [cpm] (absolute counts per min) of <em>E. coli</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.9 ± 3 (369)</td>
</tr>
<tr>
<td>PMBN</td>
<td>4.5 ± 1 (169)</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>11.0 ± 2 (410)</td>
</tr>
<tr>
<td>Neutrophils and PMBN</td>
<td>9.5 ± 1 (337)</td>
</tr>
</tbody>
</table>

NOTE. *E. coli* (OD 1.0) were incubated with R8/9 serum for 25 min in the presence of PMBN (20 μg/mL), neutrophils (1 × 10^9/mL), both neutrophils and PMBN, or none of these (control). Thereafter, the viable and nonphagocytosed *E. coli* were labeled with [3H]thymidine and underwent filter passage. Counts of *E. coli* in the filtrate, counts on the filter membrane (that is, neutrophil-associated, viable *E. coli*), and the overall sum of label incorporation are given. Values are mean ± SE of 4 independent experiments.

Figure 3. Neutrophil phosphoinositide metabolism in response to different challenges. Neutrophils (5 × 10^6/mL) were incubated with *Escherichia coli* C14 (OD 1.0) in the presence of polymyxin B nonapeptide (PMBN; 20 μg/mL), serum (10%), or both of these agents. For comparison, stimulation was performed with zymosan (5 mg/mL). Controls were sham incubated or incubated with *E. coli* + PMBN, serum + PMBN, or *E. coli* only. Extracted inositol phosphates [3H]inositol trisphosphate (IP3), [3H]inositol diphosphate (IP2), and [3H]inositol monophosphate (IP1) are collectively depicted as IPx. Diacylglycerol (DAG) formation is expressed as incorporation of labeled adenosine triphosphate. Data represent mean ± SE of 4 independent experiments each.

*Significantly different from *E. coli* + serum.

Release of granule constituents and superoxide generation. Elastase was taken as a marker for neutrophil degranulation, and enzyme activity in the cell supernatant was measured according to standard procedures [5]. Neutrophil O2 generation was measured as superoxide dismutase-inhibitable reduction of ferricytochrome c, as described elsewhere [39].

Experimental procedures. For experiments with measurement of neutrophil PAF, DAG, and phosphoinositide generation (extraction of cells and cell supernatant), burst, elastase, leukotrienes (measurement in cell supernatant), and chemotaxis, 5 × 10^6 neutrophils/mL were employed. In parallel with the preparation of human granulocytes, human serum was obtained from the same donor. For complement inactivation, serum was heated at 56°C for 30 min. Zymosan was initially opsonized, aliquoted, and stored at -20°C. If not stated otherwise, the following concentrations were chosen for the different agents on the basis of preceding pilot experiments: normal, heat-inactivated, and R8/9 serum (10% v/v each) and PMBN (20 μg/mL). For experiments with cytochalasin D, neutrophils were preincubated with 5 μg/mL cytochalasin D for 5 min. The maximum dose of PMBN was chosen to range below the threshold concentration provoking overt cell lysis. By repetitively measuring lactate dehydrogenase release via a standard colorimetric technique, this marker of cell lysis was consistently shown to range <10% of total enzyme activity (assessed in the presence of 100 μg/mL mellitin) under all experimental conditions employed.

Statistical analysis. For statistical comparison, 1-way analysis
of variance was performed. A level of $P < .05$ was considered to be significant.

**Results**

*Bacterial killing and phagocytosis.* When cultured in HBSS buffer containing 10% serum, *E. coli* C14 grew continuously to a level of 351% ± 30% of baseline within 60 min, as assessed by measurement of colony-forming units (figure 1). In the absence of serum, no bacterial growth was noted. In the presence of serum plus PMBN, a significant reduction of bacterial growth as compared with serum alone was observed, with colony-forming units ranging at 280% ± 20% (mean ± SE) of baseline after 60 min. When neutrophils were admixed to the serum-containing HBSS buffer, colony-forming unit increase was restricted to 220% ± 20% of baseline within 60 min, indicating neutrophil phagocytosis and killing activity. In the presence of serum and both PMBN and neutrophils, no bacterial growth at all occurred, but the colony-forming unit counts decreased rapidly to ~25% of baseline within 30 min. This was true for different bacterial concentrations, ranging ≥2 orders of magnitude (data not given in detail).

The PMBN-mediated increase in the killing activity of neutrophils was reproduced with the serum- and phagocytosis-resistant *E. coli* O111; in the presence of serum and both PMBN and neutrophils, colony-forming unit counts decreased markedly, whereas bacterial growth was noted under these conditions in the absence of PMBN (table 1).

**Figure 4.** Generation of leukotrienes (LTB$_4$, w-OH-LTB$_4$, and 5-HETE) and platelet-activating factor (PAF) formation (liberated and cell-bound) in response to various challenges. (A, B) Neutrophils (5 × 10$^6$/mL) were incubated with serum (10% v/v), polymyxin B nonapeptide (PMBN; 20 μg/mL), and either *Escherichia coli* C14 (OD 1.0) (A) or *E. coli* C14 adjusted to OD 1.0, 0.1, or 0.01 (B). *Significantly different from controls.* (C) Complement dependency of *E. coli*-elicited neutrophil LTB$_4$ generation. Neutrophils were incubated with *E. coli* C14 (OD 1.0) in the presence of PMBN (20 μg/mL) and either normal serum, R8/9 serum (deficient in complement factor 8/9), or heat-inactivated serum (10% v/v each) or were incubated in the absence of serum. For comparison, challenge with zymosan (5 mg/mL) and A23187 was performed (*significantly different from heat-inactivated serum). (D) Admixture of increasing doses of cytochalasin D resulted in a significant decrease of liberated LTB$_4$ (*significantly different). Neutrophils were incubated with either *E. coli* C14 (OD 1.0), PMBN (20 μg/mL), or serum (10% v/v) or with zymosan (5 mg/mL) in the presence of different concentrations of cytochalasin D for 30 min. Controls were sham incubated or incubated with serum, PMBN, or *E. coli* only. Data represent mean ± SE of 4 (A–C) or 3 (D) independent experiments each.
When heat-inactivated serum was used along with PMBN and neutrophils, the bactericidal effect of the nonapeptide plus neutrophils was fully lost, thus suggesting a central role of the complement system (figure 2). In contrast, R8/9 serum was nearly equipotent to full serum, thus excluding a major contribution of the terminal complement complex (figure 2).

When phagocytosis of bacteria was assessed by postlabeling of E. coli with [3H]thymidine after short-term incubation with neutrophils, no major loss of label (indicating bacterial uptake) or label associated with the filter membrane (including neutrophil-adherent E. coli) was observed under the experimental conditions chosen in these experiments (table 2). In contrast, in the presence of the low PMBN concentration of 2.5 μg/mL and neutrophils, a decrease of overall thymidine incorporation of nearly 50% was noted, exclusively attributable to a drop in the filtrate counts, thus indicating E. coli phagocytosis.

Neutrophil phosphoinositide metabolism. Under baseline conditions and in the presence of E. coli as well as E. coli plus PMBN, no substantial phosphoinositide metabolism was noted in the human granulocytes (figure 3). In contrast, coapplication of E. coli and serum provoked progressive accumulation of inositol phosphates in these cells, the extent of which approached that in zymosan-stimulated neutrophils. When the incubation mixture contained E. coli, serum and PMBN, this response was further enhanced ∼2-fold. Under these conditions, the maximum levels of neutrophil IP3 accumulation even slightly surpassed those in A23187-challenged granulocytes (data not given in detail). Consistent with these data, the combination of E. coli, serum, and PMBN turned out to be a most potent stimulus of human neutrophil diacylglycerol formation, with DAG levels surpassing nearly 2-fold those in response to zymosan or E. coli plus serum in the absence of PMBN (figure 3).

Lipid mediator generation. Neutrophil coincubation with E. coli in the presence of PMBN and serum provoked the synthesis of large amounts of LTβ-, α-OH-LTβ, 5-HETE, and PAF (figure 4A). LTβ levels in the supernatant plateaued within 30 min after challenge, whereas α-OH-LTβ and 5-HETE liberation progressively increased. Employment of different bacterial densities demonstrated clear dose-dependency of the lipid mediator generation (figure 4B). The leukotriene release in response to E. coli plus serum plus PMBN approximated that in response to zymosan and A23187 (figure 4C), the latter, however, being a more instantaneous trigger with rapid reversibility. It was not dependent on the presence of the terminal complement factors, as suggested by the efficacy of the R8/9 serum, but was largely blocked upon use of heat-inactivated serum and in the absence of either serum or PMBN (figure 4C). Cytochalasin D inhibited in a dose-dependent manner the neutrophil leukotriene generation elicited by E. coli in combination with R8/9 serum and PMBN, similar to that provoked by zymosan (figure 4D).

Chemotactic activity. The 4-h neutrophil migration rate toward the activated neutrophils, incubated with E. coli, PMBN, and serum, revealed a dose-dependent increase of the chemotactic index to ∼140% (OD 0.1) and ∼170% (OD 1.0), as compared with the baseline level (figure 5). Control wells,
lacking either PMBN or serum, did not provoke significant neutrophil migration.

Respiratory burst and elastase secretion. Incubation of human neutrophils with E. coli, PMBN, and serum caused a rapid degranulation, as assessed by elastase liberation and a marked respiratory burst with substantial O₂⁻ release, approximating values of zymosan-elicited oxygen radical formation upon use of OD 1.0 (figure 6). Both secretory responses plateaued after ~30 min and were significantly inhibited in the absence of PMBN and upon use of heat-inactivated serum.

Discussion

The current study employed PMBN to address the question whether its specific interaction with the LPSs at the surface of serum- and phagocytosis-resistant E. coli has a major impact on the viability of these bacteria in an environment containing both serum components and human neutrophils. As described elsewhere, some reduction of bacterial growth was noted in the presence of serum plus PMBN, as assessed in the absence of leukocytes. Leukocytes per se plus serum also effected some retardation of bacterial growth. Striking bacterial killing, however, occurred only in the presence of neutrophils plus serum components plus PMBN. Further characterization of this central finding supports a role of early complement compounds for enhanced phagocytosis of bacteria and related strong neutrophil activation, including maximally stimulated phosphoinositide metabolism, respiratory burst, degranulation, and lipid mediator generation.

Although the serum-sensitizing effect of PMBN, primarily described by Vaara and coworkers [11] and Viljanen and co-workers [40], was reproduced in the current study, this effect was rather moderate with respect to the presently employed E. coli C14, as only a slight retardation of bacterial growth was achieved upon administration of both PMBN and serum. When these components were coapplied with human neutrophils, however, bacterial growth was completely blocked, with colony-forming units being suppressed to near-zero values within 30–45 min, and this was true over the entire range of bacterial density employed. There is strong evidence that enhanced phagocytosis of the bacteria represented the predominant underlying event of this finding. First, the phagocytosis assay revealed a loss of ~45% of viable bacteria, even upon use of the very low PMBN concentration of 2.5 μg/mL in these experiments, and this loss was not due to extracellular attachment of the bacteria to the neutrophils. Second, kinetics of neutrophil-signaling events in response to E. coli plus PMBN, in particular phosphoinositide breakdown, were very similar to those upon use of zymosan, an established particulate yeast cell-wall preparation readily ingested by human neutrophils. In contrast, the A23187-induced neutrophil stimulation displayed a much more rapid time course, with partial reversibility within 15 min. And third, interference with the actin-based microfilament system by cytochalasin D, well established in its ability to block phagocytosis events [41], inhibited bacterial killing in a dose-dependent manner (data not shown) and neutrophil mediator generation, as demonstrated for LTB₄. The entire loss of any PMBN effect upon use of heat-inactivated serum, in contrast to virtual full activity when employing R8/9 serum, strongly favors a central role of early complement components in the PMBN-related enhanced phagocytosis process.

The killing of E. coli C14 in the presence of neutrophils, serum, and PMBN was paralleled by strong neutrophil activation, evident from assessment of phosphoinositide metabolism, as the predominantly preformed leukocyte signal transduction pathway. Summed concentrations of both inositol phosphate and diacylglycerol levels in E. coli serum/PMBN–activated neutrophils surpassed those in neutrophils challenged with reference concentrations of zymosan. Phosphoinositide breakdown is well known to be linked with respiratory burst, secretion of proteases (among these, neutrophil elastase is absolutely required for maximal intracellular killing of gram-negative bacteria by neutrophils [42]), generation of 5-lipoxygenase products (LTB₄ and its metabolites, 5-HETE), and PAF synthesis. All these responses were demonstrated to occur upon incubation of the neutrophils with E. coli, serum, and PMBN. The link between E. coli phagocytosis and this array of neutrophil metabolic events is further supported by the following facts: (1) Clear dose dependency of the metabolic responses on the bacterial density was noted, as displayed for LTB₄; (2) metabolic responses and bacterial killing showed the same basic features, such as, for example, dependency on the presence of both early complement components and PMBN but independence of late complement factors; (3) corresponding metabolic responses were also observed upon use of the particulate stimulus zymosan, known to forward such reactivity via phagocytosis [14]; and (4) cytochalasin D suppressed E. coli serum/PMBN–induced lipid mediator generation in a dose-dependent manner, with inhibition curves being parallel to those upon employment of zymosan as stimulus.

For the killing of serum-sensitive E. coli, it has recently been shown that the early complement components themselves may disrupt the bacterial membrane, even without the assembly of the membrane attack complex [43]. Thus, the loss of bacteria in the presence of C8/C9-depleted serum in the current study might theoretically be linked to such an effect and might not necessarily be related to neutrophil uptake and killing. This reasoning is, however, excluded by the fact that virtually no killing of E. coli C14 occurred in the absence of neutrophils. If C3 is activated on the cell surface of a pathogen, the anaphylatoxin C3a is removed and a conformational change takes place, with a thioester becoming accessible for reaction with amino or hydroxyl groups of macromolecules, thereby resulting in covalent surface binding of C3b as the most potent mechanism of opsonization [44]. Most of the activated C3 molecules, however, will become inactivated by hydrolysis, with binding
of <10% of activated C3 at the surface of the pathogen under normal conditions, and the same is true for C4. It may be speculated that PMBN-induced disorganization of the long LPSs at the outer membrane may facilitate covalent opsonin binding; however, the specific mechanisms underlying this aspect require further experimental work.

It is well in this line that PMBN strongly enhanced the neutrophil killing activity toward *E. coli* O111, with marked reduction of colony-forming units upon coapplication of neutrophils and PMBN. In contrast, as anticipated, incubation with neutrophils alone resulted in further bacterial growth. This breakthrough of bacterial resistance to neutrophil-mediated killing emphasizes a profound impact of PMBN even on serum- and phagocytosis-resistant bacterial strains that have major acceptor sites for C3 deposition other than LPSs, assumed to function as a pathogenetic factor [25].

In conclusion, the LPS-specific nontoxic agent PMBN effected a dramatic sensitization of the natively serum- and phagocytosis-resistant *E. coli* to the bactericidal efficacy of human neutrophils. Enhanced phagocytosis, dependent on early complement compounds, and strong neutrophil activation were noted as underlying events. The latter was characterized by respiratory burst and protease liberation, directly contributing to bacterial killing, and by the formation of lipid mediators such as LTβ and PAF, relevant for autocrine neutrophil activation and for chemotraction of additional neutrophils [45], as directly demonstrated in a chemotaxis assay. Such enhancement of natural leukocyte-related host defense mechanisms will avoid extracellular release of bacterial components, such as LPS, which is considered to be a disadvantageous event upon bacterial killing by antibiotics [46]. Employment of PMBN for specific interaction with the long LPS residues of smooth gram-negative bacteria, thereby optimizing opsonization, may thus offer a new therapeutic strategy in severe infections and sepsis.

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**References**

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