

## Primary and Secondary Granule Release by Polymorphonuclear Leukocytes Exposed to Peritoneal Dialysis Effluent

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**Peritoneal dialysis effluent from patients with end-stage renal failure contains a low-molecular-weight solute that inhibits the killing of phagocytosed *Staphylococcus epidermidis* by polymorphonuclear leukocytes (PMN). This observation has been investigated by using lucigenin-enhanced chemiluminescence to measure PMN NADPH oxidase activity, CD11b/CD18 expression and lactoferrin release to measure secondary granule discharge, and cellular levels of  $\beta$ -glucuronidase (EC 3.2.1.31) to measure changes in primary granules. Peritoneal dialysis effluent had no effect on the loss of intracellular  $\beta$ -glucuronidase from normal unstimulated PMN or from PMN stimulated with *S. epidermidis*. It did, however, cause a concentration-dependent (0 to 70%; vol/vol) increase in expression of CD11b/CD18 and NADPH oxidase activity. CD11b/CD18 expression increased over 20 min before starting to plateau. Release of lactoferrin by the same cells demonstrated a strong positive correlation with integrin expression ( $P < 0.001$ , Spearman's rank correlation coefficient). When dialysis effluent-treated PMN were stimulated with formyl-methionylleucylphenylalanine, integrin expression, release of lactoferrin, and NADPH oxidase activity were greater than in PMN treated with formyl-methionylleucylphenylalanine alone. Under these conditions, a concentration-dependent increase in CD11b/CD18 and lactoferrin release were observed only at a concentration between 0 and 30% (vol/vol) dialysis effluent, while a concentration-dependent increase in oxidase activity was seen at a concentration between 0 and 70% (vol/vol). The results suggest that dialysis effluent does not affect PMN primary granule release but does cause increased release of secondary granules and an increase in NADPH oxidase activity in both unstimulated and stimulated PMN.**

The use of continuous ambulatory peritoneal dialysis for the treatment of end-stage renal failure is frequently complicated by peritonitis, which is diagnosed when the dialysis fluid becomes cloudy as a result of an influx of polymorphonuclear leukocytes (PMN). In spite of this vigorous cellular response, infection is difficult to eradicate even by the use of antibiotics to which the organisms are sensitive. It appears that PMN in dialysis fluid are unable to function normally. Factors including pH (7, 11, 20), osmolality (7), and lack of opsonins (15) are thought to impair ingestion of organisms, while a low-molecular-weight toxin in spent dialysis fluid inhibits intracellular microbicidal function (6, 13). The microbicidal function of PMN depends largely upon their ability to discharge primary and secondary granules. Secondary degranulation causes the translocation of receptors and cytochrome  $b_{558}$  to the plasma membrane, while primary granules release myeloperoxidase and hydrolytic and proteolytic enzymes into the phagocytic vacuole. Since peritoneal dialysis effluent (PDE) impairs the microbicidal action of PMN, we have investigated the effect of PDE upon granule discharge and NADPH oxidase activity.

### MATERIALS AND METHODS

**Preparation of PMN.** Human peripheral blood PMN were prepared by standard methods (3, 21). Fresh venous blood was added to EDTA (dipotassium salt) to give a final concentration of 3.5 mM. Erythrocytes were sedimented on dextran, and the leukocyte-rich plasma was further purified over a Ficoll gradi-

ent (lymphocyte separation medium; Flow Laboratories, Hertfordshire, United Kingdom). The PMN-rich pellet was subjected to hypotonic lysis to remove the remaining erythrocytes before being washed twice in HEPES buffer (pH 7.4) containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 145 mM sodium chloride, 5 mM potassium chloride, 5 mM glucose, and 0.1% (wt/vol) albumin. Purification of cells by this method routinely gave preparations of >99% viability as assessed by trypan blue exclusion and of >97% purity as assessed by examination of stained cytospin preparations. Cells were counted in a hemocytometer and suspended at a concentration of  $10^7$ /ml.

**PDE.** Eight PDEs (1.36% [wt/vol] glucose) (Dianeal; Baxter Travenol Inc., Chicago, Ill.) were obtained from patients receiving continuous ambulatory peritoneal dialysis after an intraperitoneal dwell of 4 h. All patients had been established on continuous ambulatory peritoneal dialysis for more than 2 months, were not suffering infection, and had not received antibiotic therapy over the preceding 4 weeks. The PDE was stored at  $-70^\circ\text{C}$  until required. Before use, the PDE was filtered through a 0.2- $\mu\text{m}$ -pore-size membrane and the pH was adjusted to 7.4 by the addition of HEPES to give a final concentration of 20 mM. Creatinine and urea concentrations of fluids were determined by autoanalysis; endotoxin concentrations were determined by using the *Limulus* amoebocyte lysate assay (LAL-Sigma Diagnostic Kit; Sigma Chemical Co., Poole, Dorset, United Kingdom).

**Measurement of primary granule release. Assay of  $\beta$ -glucuronidase.** In the absence of cytochalasin B, even a potent stimulus of degranulation such as phagocytosis of opsonized bacteria causes very little discharge of primary granule contents into the external medium. However, primary granule

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enzymes, such as  $\beta$ -glucuronidase, are denatured after release into the phagocytic vacuole, and this provides a convenient method of measuring primary degranulation.

Intracellular loss of  $\beta$ -glucuronidase from PMN was measured by using a diagnostic kit (Sigma). The basic reaction mixture contained HEPES buffer (pH 7.4), 1 mM  $\text{CaCl}_2$ , 0.7 mM  $\text{MgCl}_2$ , and 0.1% (wt/vol) albumin. *S. epidermidis*, preopsonized with 10% (vol/vol) pooled human serum, was added to give a final concentration of  $2 \times 10^7$ /ml. Dialysis effluents were routinely used at 50% (vol/vol). The reaction was initiated by the addition of PMN to give a final concentration of  $4 \times 10^6$  cells per ml. Tubes were incubated at  $37^\circ\text{C}$  with constant rotation. Aliquots (equivalent to  $10^6$  PMN) were removed at various time points, and the reaction was terminated by the immediate addition of ice-cold HEPES buffer. PMN were washed twice in cold buffer before the cell pellet was lysed by the addition of buffer containing 0.5% (vol/vol) Triton X-100. After being sonicated for 60 s (Rapids 50 Ultrasonic Disintegrator; Ultrasonics, Shipley, England), samples were centrifuged to remove cell debris and 200  $\mu\text{l}$  of the supernatant was assayed for the presence of  $\beta$ -glucuronidase. Under these conditions, complete disruption of PMN granules was achieved (data not shown). The results are expressed as a percentage of the total  $\beta$ -glucuronidase in unstimulated PMN (at time zero).

**Measurement of secondary granule release. (i) Assay of CD11b/CD18.** The integrin CD11b/CD18 is involved in the phagocytosis of bacteria; consequently, its expression is difficult to measure in the presence of particulate stimuli. For this reason, in the following experiments, the soluble chemotactic peptide formyl-methionylleucylphenylalanine (fMLP) was used as a stimulus.

The expression of CD11b and CD18 was determined by flow cytometry by using a direct, double-labelling immunofluorescence technique (4). The basic reaction mixture contained HEPES buffer (pH 7.4), 1 mM  $\text{CaCl}_2$ , 0.7 mM  $\text{MgCl}_2$ , and 0.1% (wt/vol) albumin. PDEs were used routinely at 50% (vol/vol) in the reaction mixture; fMLP was used routinely at a final concentration of 1  $\mu\text{M}$ . The reaction was initiated by the addition of PMN to give a final concentration of  $10^6$  cells per ml. Samples (equivalent to  $5 \times 10^5$  PMN) were removed at various times and immediately centrifuged at 1,500 rpm ( $400 \times g$ ;  $4^\circ\text{C}$ ) for 5 min. Supernatants were retained for the measurement of lactoferrin. The cell pellet was washed twice in ice-cold HEPES buffer and resuspended in 1.0 ml of buffer. Five microliters of fluorescein isothiocyanate-conjugated anti-CD18 (Dako), phycoerythrin-conjugated anti-CD11b (Becton Dickinson), or isotype-matched antibody to keyhole limpet hemocyanin (Simultest; immunoglobulin G1-fluorescein isothiocyanate and immunoglobulin G2a-phycoerythrin; Becton Dickinson) was added, and the cells were incubated for 30 min at  $4^\circ\text{C}$ . Samples were centrifuged and washed twice in HEPES buffer. CD11b and CD18 antigen expression was determined by using a Becton Dickinson FACScan, and the results are presented as mean fluorescence.

**(ii) Assay of lactoferrin.** Determination of the amount of lactoferrin released by PMN was performed with cell supernatants generated during the measurement of CD11b/CD18 expression. The assay used was an enzyme-linked immunosorbent assay described in detail elsewhere (4). The results are presented as nanomoles of lactoferrin per  $10^6$  cells.

**Measurement of superoxide anion production.** Superoxide production by PMN was determined by lucigenin-enhanced chemiluminescence in a Bio-Orbit model 1251 luminometer (12). The reaction mixture (500  $\mu\text{l}$ ) contained 25  $\mu\text{M}$  lucigenin, HEPES buffer (pH 7.4), 1 mM  $\text{CaCl}_2$ , and 0.7 mM  $\text{MgCl}_2$ . PDEs were routinely used at a concentration of 50% (vol/vol);

TABLE 1. Creatinine, urea, and endotoxin concentrations in PDE

Concn determination	Creatinine ( $\mu\text{M}$ )	Urea (mM)	Endotoxin (pg/ml)
Mean $\pm$ SEM	$900 \pm 70$	$18.18 \pm 1.56$	$71.25 \pm 9.22$
Range (minimum–maximum)	707–1,290	14.0–25.4	57–114

fMLP was used at a final concentration of 1  $\mu\text{M}$ . PMN, to give a final concentration of  $10^6$ /ml, were added to the reaction mixture immediately before chemiluminescence was measured.

**Statistical analysis.** All data are given as the mean  $\pm$  standard error of the mean (SEM). Analysis was by the Wilcoxon signed rank test and Spearman's rank correlation.

## RESULTS

Mean creatinine, urea, and endotoxin concentrations in the dialysis effluents used are presented in Table 1. The inclusion of these compounds in the assays at the maximum concentrations encountered in PDE did not affect the results obtained (data not shown). Furthermore, pH-corrected unused dialysis fluid (50%, vol/vol) did not effect PMN degranulation or NADPH oxidase activity (data not shown).

**Primary granule release.** PDEs contained no intrinsic  $\beta$ -glucuronidase (data not shown) and did not induce a significant loss of the enzyme from PMN primary granules over a period of 40 min (Fig. 1). When preopsonized *S. epidermidis* was used to stimulate cells, the amount of intracellular  $\beta$ -glucuronidase gradually fell to 35% of its original level over 40 min. The incorporation of dialysis effluent into the assay had no influence upon enzyme loss (Fig. 1).

**Secondary granule release.** The effect of PDE on CD11b

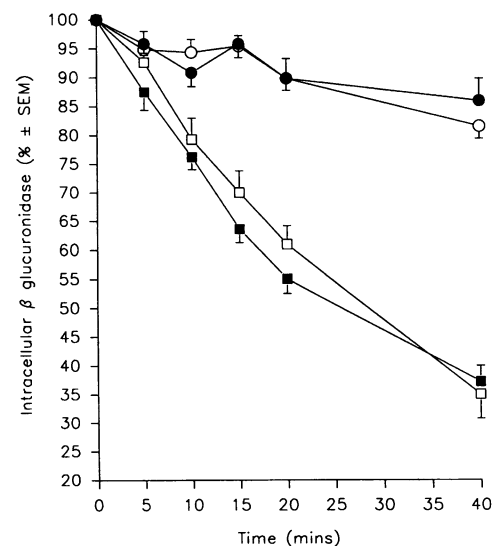


FIG. 1. Intracellular levels of  $\beta$ -glucuronidase in the presence (filled symbols) and absence (open symbols) of PDE (50%, vol/vol). Cells were either at rest (circles) or stimulated with preopsonized *S. epidermidis* (squares). One hundred percent  $\beta$ -glucuronidase represents  $10.20 \pm 0.45 \text{ U}/10^6 \text{ PMN}$ , where 1 U of  $\beta$ -glucuronidase activity will liberate 1  $\mu\text{g}$  of phenolphthalein from phenolphthalein glucuronic acid per h at  $56^\circ\text{C}$ . The results are the mean  $\pm$  SEM of 16 experiments (eight dialysis effluents, each tested on two separate donor PMN).

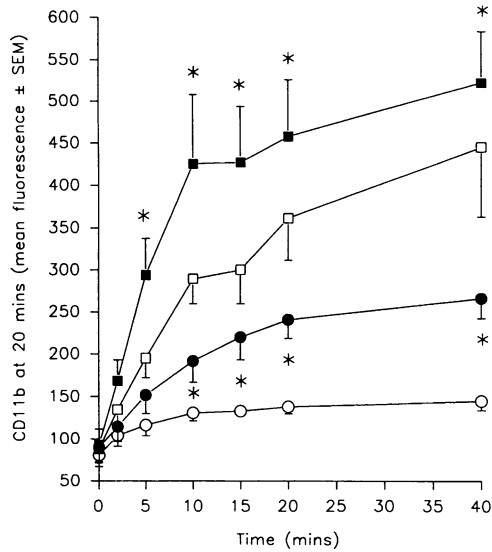


FIG. 2. The expression of CD11b in the presence (filled symbols) and absence (open symbols) of PDE (50%, vol/vol) over time. PMN were either unstimulated (circles) or fMLP stimulated (squares). The results are the mean ± SEM of 16 experiments (eight dialysis effluents, each tested on two separate donor PMN). An asterisk (\*) indicates a statistical difference from results with a buffer control ( $P < 0.05$ ; Wilcoxon signed rank test).

expression by normal PMN is shown in Fig. 2. PDE with no second stimulus caused low-level but significant expression of CD11b with respect to control ( $P < 0.05$ ; Wilcoxon signed rank). When cells were also stimulated with fMLP, the presence of PDE significantly increased CD11b expression above that of the control ( $P < 0.05$ ; Wilcoxon signed rank). The concentration response relationship between CD11b expression and PDE for stimulated and unstimulated PMN is shown

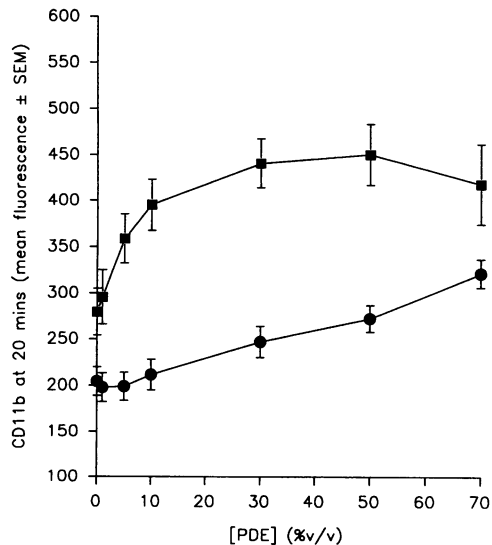


FIG. 3. The expression of CD11b with increasing concentrations of PDE (0 to 70%, vol/vol). Cells were either unstimulated (●) or stimulated with fMLP (■). The results are the mean ± SEM of 16 experiments (eight dialysis effluents, each tested on two separate donor PMN).

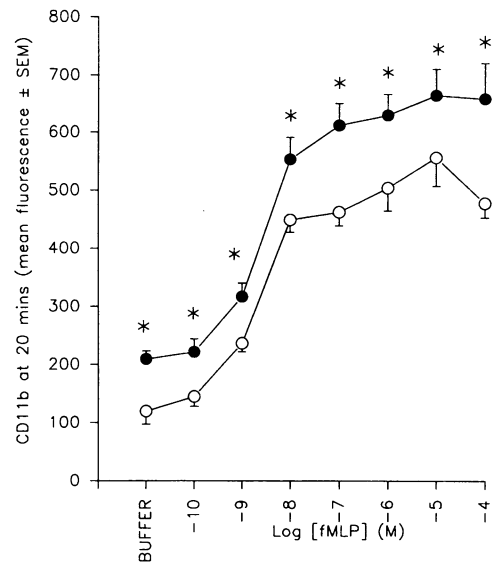


FIG. 4. The expression of CD11b by PMN stimulated with various concentrations of fMLP in the presence (●) and absence (○) of PDE (50%, vol/vol). The results are the mean ± SEM of 16 experiments (eight dialysis effluents, each tested on two separate donor PMN). An asterisk (\*) indicates a significant difference from the results with a buffer control ( $P < 0.05$ ; Wilcoxon signed rank test).

in Fig. 3. Figure 4 shows the dose-response relationship between fMLP and CD11b expression and demonstrates that the presence of PDE does not affect this relationship. Lactoferrin release (Fig. 5) and CD18 expression (data not shown) in the presence of PDE showed the same time course and concentration response relationship as CD11b expression,

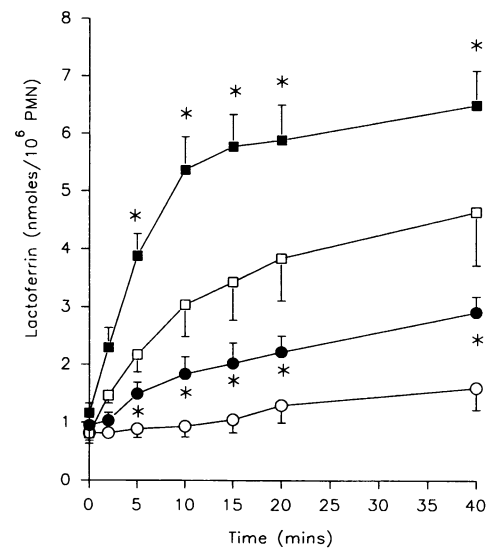


FIG. 5. The release of lactoferrin in the presence (filled symbols) and absence (open symbols) of PDE (50%, vol/vol) over time. PMN were either unstimulated (circles) or fMLP stimulated (squares). The results are the mean ± SEM of 16 experiments (eight dialysis effluents, each tested on two separate donor PMN). An asterisk (\*) indicates a statistical difference from the results with a buffer control ( $P < 0.05$ ; Wilcoxon signed rank test).

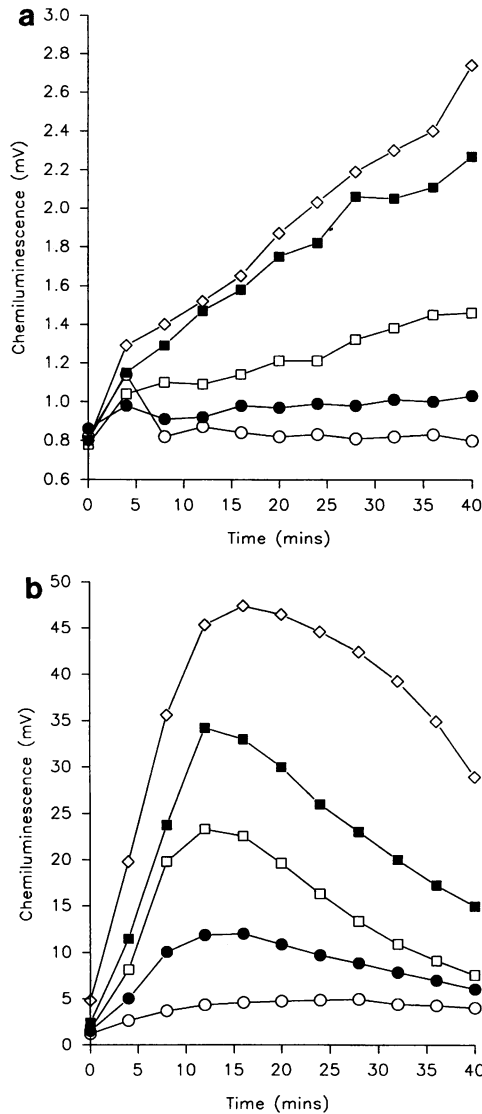


FIG. 6. Lucigenin-enhanced chemiluminescence of unstimulated (a) and fMLP-stimulated (b) PMN in the presence of increasing concentrations of dialysis effluent, i.e., 0 (○), 10 (●), 30 (□), 50 (■), and 70% (◇; vol/vol). The results show a typical single experiment representative of 16 experiments (eight dialysis effluents, each tested on two separate PMN).

both when PDE was present alone and in the presence of PDE with fMLP stimulation. Statistically, there was a strong positive correlation between integrin expression and lactoferrin release ( $P < 0.001$ ; Spearman's rank correlation).

**NADPH oxidase activity.** PDE alone produced a slow, low-level, but concentration-dependent increase in chemiluminescence that was still increasing after 40 min of incubation (Fig. 6a). Dialysis effluent also had a concentration-dependent effect upon fMLP-stimulated chemiluminescence that was maximal at approximately 12 min before starting to decline (Fig. 6b).

When CD11b expression and oxidase activity were monitored over time in the same PMN, stimulation with PDE (data not shown), fMLP (data not shown), or fMLP in the presence of PDE (Fig. 7) resulted in the peak rate of CD11b expression

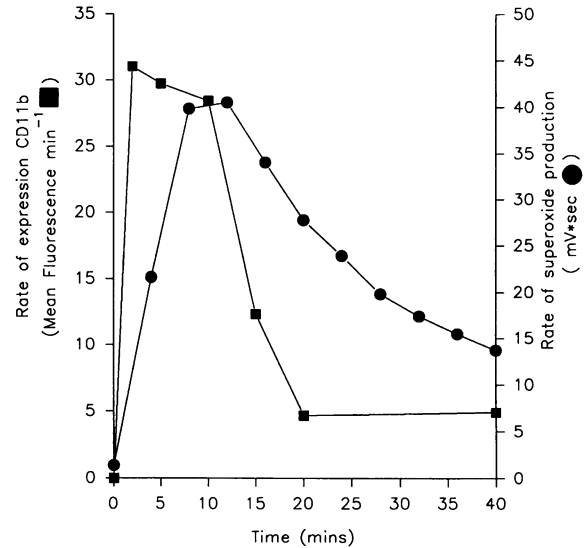


FIG. 7. The rate of CD11b expression (■) compared with the rate of chemiluminescence (●) after fMLP stimulation of the same PMN exposed to the same dialysis effluent. The results show a typical single experiment representative of eight experiments (eight dialysis effluents, each tested on PMN from a single donor).

(maximal at 2 min) preceding the peak rate of chemiluminescence (maximal at 12 min; Fig. 7).

## DISCUSSION

The effect of PDE upon normal PMN function presents the paradox that PDE increases production of oxygen radicals in response to fMLP and opsonized microorganisms (5) while it also impairs intracellular killing of bacteria (13). The factor(s) responsible for these two effects can be isolated in a single low-molecular-weight fraction by size-exclusion chromatography (6). Circulating and peritoneal PMN from patients treated by continuous ambulatory peritoneal dialysis demonstrate similar abnormalities (14), suggesting that the factor(s) responsible for these effects are circulating toxin(s).

Increased production of oxygen radicals and reduced killing of bacteria could be explained by an effect of PDE upon primary granule discharge. Fusion of the primary granules with a phagocytic vacuole is responsible for the delivery of myeloperoxidase and other antimicrobial enzymes to the vacuole. Stimulation of PMN with opsonized bacteria causes the cell to discharge small amounts of primary granule enzymes (less than 10% of the intracellular content) into the external medium. This is accompanied by a greater loss of cellular enzyme that is thought to be due to denaturation by hydrogen peroxide within the phagocytic vacuole. The data presented in Fig. 1 demonstrate that PDE, either alone or in combination with fMLP, does not affect the loss of  $\beta$ -glucuronidase from PMN, suggesting that primary degranulation remains intact when these cells are exposed to dialysis effluent.

In contrast to the lack of effect on primary degranulation, PDE had a considerable effect on the release of secondary granules from PMN. Both the integrin CD11b/CD18 and lactoferrin are contained within the secondary granules (10, 16, 19) which discharge to the cell surface when PMN are stimulated. It is not, therefore, surprising that integrin expression and lactoferrin release occur in parallel. These two markers showed that PDE alone caused low-level secondary granule



discharge that was accompanied by a similar, slow, low-level stimulation of the NADPH oxidase. Stimulation of the oxidase may be linked to secondary granule discharge by translocation to the cell surface of cytochrome  $b_{558}$ , which is contained within the secondary granules and is also a component of the NADPH oxidase (16, 17).

PDE enhanced secondary granule release and oxidase activity by fMLP-stimulated PMN. The peak rate of secondary degranulation preceded the peak rate of superoxide anion production by some 10 min (Fig. 7). This may be due to increased expression of fMLP receptors contained within the secondary granules (8). Increased fMLP receptor expression has been proposed as the possible mechanism by which the fungal metabolite cytochalasin B augments fMLP-induced superoxide generation (1). Increased fMLP receptor expression is, however, also associated with a decrease in the affinity of the receptor (9). This might be expected to shift the concentration response curve for fMLP; however, Fig. 4 shows that this is not the case.

Not only did the rate of fMLP-induced secondary degranulation peak earlier than the rate of fMLP-stimulated superoxide production, but there was also a difference in the dose-response relationship to PDE (Fig. 3). Secondary degranulation reached a plateau above a PDE concentration of 30% (vol/vol), while fMLP-stimulated oxidase activity continued to increase up to 70% (vol/vol), which was the maximum PDE concentration possible. Furthermore, there was a 5-fold increase in oxidase activity at 20 min compared with only a 1.5-fold increase in integrin expression in the same cells. PDE may therefore prime the oxidase response to fMLP not only by translocating fMLP receptors and cytochrome  $b_{558}$  to the cell surface but also by affecting the assembly of other components of the enzyme that are not contained within secondary granules. Priming is reminiscent of tumor necrosis factor, which also causes rapid secondary degranulation (18) and, at high concentrations, stimulates low-level oxidase activity (2). However, there was no detectable tumor necrosis factor in the PDEs used in this work (data not shown).

The experiments described have failed to explain the apparent paradox of increased oxygen radical production associated with decreased microbicidal activity of normal PMN in the presence of PDE. Although primary degranulation appears to be unaffected by PDE, the possibility remains that myeloperoxidase may be selectively denatured by factor(s) in effluent. This would prevent formation of hypochlorous acid and the substances derived from it that have a high antibacterial potential and presumably result in reduced killing. This possibility is currently being investigated.

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