Polymorphonuclear Leukocyte Oxidative Burst Is Enhanced in Patients With Chronic Renal Insufficiency

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ABSTRACT
Previous reports that polymorphonuclear leukocyte (PMN) function is impaired in hemodialysis patients do not differentiate between effects of dialysis and of uremia. The hypothesis that chronic renal insufficiency impairs PMN function was tested. Phagocytosis and oxidative burst were measured in PMN from patients with varying degrees of chronic renal insufficiency (creatinine clearance, 6 to 35 mL/min per 1.73 m²) and normal subjects. The ability of tumor necrosis factor-α (TNF-α) to prime the oxidative burst was also assessed. Phagocytosis of Staphylococcus aureus and basal H₂O₂ and O₂⁻ release by PMN did not differ between normal subjects and patients with chronic renal insufficiency. However, the oxidative burst stimulated by S. aureus and formyl-Met-Leu-Phel, but not phorbol myristate acetate, was significantly enhanced in PMN from patients with chronic renal insufficiency. The increase in formyl-Met-Leu-Phel-stimulated oxidative burst correlated significantly with the level of renal function. TNF-α significantly increased S. aureus-induced H₂O₂ production in normal PMN, but not in PMN from patients with chronic renal insufficiency. These data indicate that chronic renal insufficiency does not impair PMN phagocytosis and oxidative burst. To the contrary, it enhances receptor-mediated oxidative burst. The inability of TNF-α to further enhance the oxidative burst suggests that PMN exist in a primed state in patients with chronic renal insufficiency.

Key Words: Neutrophil, oxygen radicals, phagocytosis, priming, uremia

Hemodialysis patients have an increased risk of infection (1) and of death from infectious complications (2); however, the mechanism behind this increased risk remains to be determined. Circulating polymorphonuclear leukocytes (PMN) are a necessary component of the immune response that protects against bacterial infections. In this response, external agonists act through plasma membrane receptors to coordinate a complex series of actions by PMN, including their adherence to the vascular endothelium at the site of infection, transmigration across the vascular wall, chemotaxis toward and phagocytosis of bacteria, and killing of ingested bacteria by reactive oxygen metabolites and lysosomal enzymes (3,4). Because of the importance of PMN in defending against invading microorganisms, many of their functions, particularly those directly related to killing bacteria, have been studied in hemodialysis patients (5-9). Those studies have produced highly variable results, with phagocytosis and the oxidative burst being reported as increased, normal, or decreased. Although some of the disparity between results of different studies may be explained by differences in methodology, a major impediment in assessing PMN function in hemodialysis patients is an inadequate delineation of the effects of the uremic state.

Relatively few studies have focused on PMN function in patients with chronic renal insufficiency before the initiation of dialysis. Vanholder et al. (10) and Lucchi et al. (7) found that the stimulated PMN oxidative burst decreased as renal function decreased. However, Vanholder et al. (10) measured oxidative burst indirectly as 14CO₂ production from glucose-1-14C over 60 min in response to latex and zymosan particles. Thus, their findings may reflect the total metabolic capacity of uremic PMN rather than their ability to produce a short-lived oxidative burst. Lucchi et al. (7) assessed the oxidative burst by measuring the increase in luminol-enhanced chemiluminescence stimulated by the phagocytosis of opsonized zymosan. Chemiluminescence methods may provide different results than methods that directly measure oxygen radical production. For example, PMN from hemodialysis patients stimulated with phorbol myristate acetate (PMA) demonstrate normal O₂⁻ release, but reduced chemiluminescence compared with normal subjects (11).

To assess the effect of uremia on PMN functions, we measured the ability of PMN to phagocytose Staphylococcus aureus and produce H₂O₂ and to release O₂⁻ in response to PMA and formyl peptides in patients with varying degrees of renal function. We also determined the ability of the cytokine tumor necrosis factor-α (TNF-α) to prime PMN H₂O₂ production. Our results demonstrate that neutrophils from patients with chronic renal insufficiency have an enhanced oxidative burst in response to receptor-mediated stimuli,
but not to PMA. However, the ability of TNF-α to prime H$_2$O$_2$ production in response to phagocytosis was impaired in uremic PMN. These results suggest that PMN exist in a primed state in patients with chronic renal insufficiency.

**METHODS**

**Patients**

Twenty-six patients with varying degrees of renal insufficiency participated in the study. The study was approved by the Human Studies Committee of the University of Louisville, and all subjects gave informed consent before participating in the study. Exclusion criteria included: a history of malignancy or hematologic diseases, other than the anemia of renal failure; recent infection; and prescription of medications such as antibiotics, corticosteroids, or immunosuppressive agents. The patients included 17 women and 9 men with a mean age of 54 yr (range, 29 to 88 yr). The cause of their renal insufficiency included diabetes (N = 11), hypertension (N = 7), polycystic kidney disease (N = 2), hydronephrosis (N = 1), chronic pyelonephritis secondary to reflux (N = 1), and glomerulonephritis (N = 1), and was unknown in 3 patients. Serum creatinine concentrations ranged from 1.9 to 15.3 mg/dL, and creatinine clearances, calculated by the method of Cockcroft and Gault (12), ranged from 6 to 35 mL/min per 1.73 m$^2$. Seventeen healthy subjects were enrolled as normal controls. A single blood sample was obtained from each subject. After needle insertion, the first 3 mL of blood withdrawn was discarded to avoid blood components activated during venipuncture. Blood was then drawn into tubes containing the appropriate anticoagulant for the various assays of PMN function as described below. Blood samples were also obtained for the determination of white blood cell count and differential and serum creatinine by standard clinical laboratory methods. In all cases, white blood cell and neutrophil counts were in the normal range. Blood was also obtained from a normal control subject. One or more patient samples and a control sample were then processed in parallel through the various assays.

**PMN Isolation**

PMN were isolated from EDTA-anticoagulated blood by single-step gradient centrifugation (Polyprep; Nycomed, Oslo, Norway) at room temperature. The mononuclear cell layer was aspirated and discarded. The PMN layer was recovered, contaminating erythrocytes were removed by hypotonic lysis, and the PMN were washed and resuspended in buffer containing calcium and magnesium. The cell count was adjusted to 2.5 × 10$^6$ cells/mL.

**Phagocytosis and H$_2$O$_2$ Production**

Phagocytosis of *S. aureus* and the subsequent production of H$_2$O$_2$ was measured in whole blood by a modification of the flow cytometric assay described by Trinkle and colleagues (13). Briefly, formalin-fixed *S. aureus* of the Cowan I strain (Pansorbin Standardized; Behring-Calbiochem, San Diego, CA) was labeled with propidium iodide (Sigma Chemical Co., St. Louis, MO) by resuspending Pansorbin Standardized in buffer (pH = 7.2) containing 300 mM propidium iodide and incubating the mixture at room temperature for 1 h with constant agitation. The bacteria were then washed and resuspended in buffer. Phagocytosis of *S. aureus* and the subsequent H$_2$O$_2$ production were determined by the addition of 50 μL of 0.5 mM 2',7'-dichlorofluorescin diacetate (Molecular Probes, Eugene, OR) to 0.5 mL of whole blood and incubation in a shaking water bath for 10 min at 37°C. Fifty microliters of blood was then sampled before and 10 min after the addition of 50 μL (approximately 10$^8$ bacteria) of propidium iodide-labeled *S. aureus*. After lysis of the erythrocytes, the remaining cells were washed with buffer and fixed in 1% paraformaldehyde. Samples were analyzed by flow cytometry (Epics Profile II; Coulter, Hialeah, FL) within 1 h of processing. PMN were selectively gated for analysis on the basis of forward versus right angle light scatter. Phagocytosis was measured as the fluorescence intensity of light emitted by propidium iodide-labeled bacteria. Oxidative burst was measured as the fluorescence intensity of light emitted by dichlorofluorescin, stoichiometrically produced by the action of H$_2$O$_2$ on the nonfluorescing 2',7'-dichlorofluorescin. Light emission was measured on 5,000 neutrophils per sample, and the data were stored in the log mode. The mean channel number of fluorescence intensity was used as a quantitative index of phagocytosis and H$_2$O$_2$ production. The flow cytometer was calibrated before the analysis of each set of samples with Standard-Brite beads (Coulter).

**O$_2^-$ Production**

O$_2^-$ production was determined in isolated PMN by the measurement of the reduction of ferricytochrome-C (14). PMN (final concentration, 2 × 10$^6$ cells/mL) were resuspended in buffer containing calcium, magnesium, and 1 mg/mL ferricytochrome-C. O$_2^-$ production was stimulated by the addition of 10$^{-7}$ M formyl-methionyl-leucyl-phenylalanine (FMLP) or 100 nM PMA for 10 min at 37°C. Incubation was stopped by placing the tubes on ice, and the cells were pelleted by centrifugation at 4°C. The amount of O$_2$- produced was quantified by measuring the change in absorbance of the supernatant at 550 nm and was expressed as nanomoles of O$_2$- produced per 10$^6$ cells with an extinction coefficient of 2.1 × 10$^4$ M$^{-1}$ cm$^{-1}$. All experiments were performed in duplicate.

**TNF-α Priming of Phagocytosis and H$_2$O$_2$ Generation**

The ability of TNF-α to prime PMN phagocytosis and H$_2$O$_2$ production was assessed by the incubation of whole blood with and without 200 IU/mL recombinant human TNF-α (Genzyme, Cambridge, MA) for 10 min at 37°C. Phagocytosis of *S. aureus* and the subsequent H$_2$O$_2$ production were then determined as described above.

**Data Analysis**

The dependence of PMN phagocytosis and the oxidative burst in patients with chronic renal insufficiency on the degree of renal function was assessed by linear regression analysis. Because Vanholder and colleagues (10) excluded diabetic subjects from their study population and because it has been suggested that diabetes may affect PMN function (15), multiple linear regression was also used to examine the dependence of PMN function on creatinine clearance in the presence and absence of diabetes. Phagocytosis and the oxidative burst in PMN of patients with chronic renal insufficiency were compared with those in PMN from normal subjects by t test for unpaired data. The relative ability of TNF-α to prime H$_2$O$_2$ production in PMN from patients with chronic renal insufficiency and from normal control subjects was assessed by a two-way analysis of variance, with posthoc
testing by the Student-Newman-Keuls method. All statistical analyses were performed with the SigmaStat statistical package (Jandel Scientific, San Rafael, CA).

RESULTS

To determine if PMN from patients with chronic renal insufficiency have impaired function, phagocytosis and phagocytosis-induced H2O2 production were measured. Phagocytosis of S. aureus was not significantly different between normal subjects and patients with chronic renal insufficiency (Table 1). In contrast, H2O2 production after phagocytosis was significantly greater in the patient group than in normal subjects (P < 0.001; Table 1). This difference remained significant when H2O2 production was normalized to the level of phagocytosis. There was no difference in unstimulated H2O2 production between the two groups. Diabetes mellitus has been reported to affect PMN functions (15); however, there was no difference in the level of phagocytosis or H2O2 production between diabetic and nondiabetic patients (P > 0.863), and the inclusion of diabetes as a covariate did not affect the results of the comparison between normal subjects and patients with renal insufficiency. To determine if the increased H2O2 production was related to the level of renal function, the two were compared by linear regression analysis. There was no correlation between the level of phagocytosis (P = 0.359) or H2O2 production (P = 0.093) and the level of renal function. The inclusion of diabetes in a multiple linear regression did not change the finding that phagocytosis and H2O2 production did not correlate with level of renal function.

Extracellular fMLP stimulates the oxidative burst by interacting with guanine nucleotide binding protein (G protein)-coupled receptors, whereas PMA bypasses transmembrane signaling pathways by directly stimulating protein kinase C. Receptor-stimulated and PMA-stimulated oxidative burst were compared in patients and control subjects by measuring O2− release from isolated PMN. As shown by the data in Table 2, unstimulated and PMA-stimulated O2− release was not significantly different between the two groups (P = 0.200 and P = 0.744, respectively). On the other hand, PMN from patients with chronic renal insufficiency released significantly more O2− after stimulation with fMLP than did PMN from control subjects (P = 0.022; Table 2). Additionally, linear regression analysis revealed that fMLP-stimulated O2− production increased significantly (r = 0.504; P = 0.023) as creatinine clearance decreased (Figure 1). There was no correlation between resting (P = 0.888) or PMA-stimulated (P = 0.327) PMN O2− release and calculated creatinine clearance. Again, the presence or absence of diabetes did not change these findings.

An important component of PMN responses is the ability of their functions to be enhanced or primed during infection. TNF-α is a cytokine that participates in PMN priming (16). Because PMNs from patients with chronic renal insufficiency demonstrated an enhanced oxidative burst, we examined if this activity could be further primed by TNF-α. Whole-blood PMN were incubated with 200 IU/mL TNF-α for 10 min before the stimulation of H2O2 production by phagocytosis. Preliminary studies demonstrated this concentration of TNF-α, and time of exposure provided an optimal priming of PMN from normal individuals. As shown in Figure 2A, the preincubation of PMN from normal subjects with TNF-α resulted in a significant increase in S. aureus–stimulated H2O2 production (P = 0.006), whereas phagocytosis was not increased significantly (P = 0.072). In contrast, PMN from patients with chronic renal insufficiency preincubated with TNF-α failed to significantly increase either phagocytosis (P = 0.859) or H2O2 production (P = 0.769; Figure 2B). By two-way analysis of variance, the increase in S. aureus–stimulated H2O2 production after priming by TNF-α differed significantly between PMN from normal subjects and those from patients with chronic renal insufficiency (P = 0.003).

DISCUSSION

The purpose of this study was to establish a frame of reference with which to interpret alterations in PMN function of chronic hemodialysis patients. PMN function has been studied extensively in chronic hemodialysis patients for two reasons. First, they are reported to have an increased susceptibility to, and an increased mortality from, infections (1,2). Second, changes in PMN function have been used as a measure of the biocompatibility of hemodialysis membranes (3). However, PMN function in patients being

<table>
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<tr>
<th>Parameter</th>
<th>Normal</th>
<th>Chronic Renal Insufficiency</th>
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<tbody>
<tr>
<td>Phagocytosis (mcf)</td>
<td>13.3 ± 6.2 (N = 16)\textsuperscript{b}</td>
<td>15.9 ± 6.7 (N = 23)</td>
</tr>
<tr>
<td>H2O2 Production (mcf)</td>
<td>0.89 ± 0.52 (N = 17)</td>
<td>1.15 ± 0.74 (N = 24)</td>
</tr>
<tr>
<td>Resting</td>
<td>31.1 ± 11.1 (N = 17)</td>
<td>49.1 ± 18.0 (N = 24)</td>
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<td>S. aureus–stimulated</td>
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\textsuperscript{a}Mean channel of fluorescence intensity

\textsuperscript{b}Data are presented as mean ± SD for N observations.

\textsuperscript{c}Significantly different than normal (P < 0.001)

TABLE 1. PMN phagocytosis and resting and S. aureus–stimulated H2O2 production in whole blood from normal subjects and patients with chronic renal insufficiency

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TABLE 2. Resting and fMLP- and PMA-stimulated $O_2^-$ production in isolated PMN from normal subjects and patients with chronic renal insufficiency

<table>
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<tr>
<th>Type of Production</th>
<th>Normal</th>
<th>Chronic Renal Insufficiency</th>
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<tbody>
<tr>
<td>Resting</td>
<td>1.53 ± 0.93 (N = 15)$^a$</td>
<td>2.05 ± 1.30 (N = 20)</td>
</tr>
<tr>
<td>fMLP-stimulated</td>
<td>9.4 ± 3.8 (N = 15)</td>
<td>13.7 ± 6.1 (N = 20)$^b$</td>
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<tr>
<td>PMA-stimulated</td>
<td>25.0 ± 9.3 (N = 15)</td>
<td>24.2 ± 3.3 (N = 20)</td>
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$^a$ Data are presented as mean ± SD for N observations.
$^b$ Significantly different than normal (P = 0.022).

Initiated onto dialysis is not firmly established. Thus, it was necessary to examine the effect of uremia, alone, on PMN function in order to interpret data examining the effects of hemodialysis.

This study identifies specific abnormalities of PMN function in patients with chronic renal insufficiency. We found that patients with various levels of chronic renal insufficiency have an enhanced PMN oxidative burst capacity (Tables 1 and 2). This increased activity appears to be independent of the cause of renal insufficiency. In particular, patients with diabetes mellitus demonstrated the same abnormality as patients with other causes of renal disease. There was a significant correlation between $O_2^-$ release stimulated by fMLP and calculated creatinine clearance (Figure 1), suggesting that the degree of enhancement of the oxidative burst may be related to the level of renal function. However, phagocytosis-stimulated $H_2O_2$ production did not correlate with the calculated creatinine clearance. Although the discrepancy in results may be related to differences in the assay systems, the inconsistent results do not allow us to state unequivocally that altered PMN function is related to the level of glomerular filtration.

Our findings are in contrast to those of two previous studies that examined the PMN oxidative burst in patients with renal insufficiency (7, 10). Significant methodologic differences make it difficult to reconcile the disparate findings, because our studies differ from
used to detect and the agonist used to stimulate the oxidative burst. Lucchi et al. (7) determined the oxidative burst by measuring luminol-enhanced chemiluminescence. They found a decrease in chemiluminescence in patients with GFR less than 10 mL/min compared with normal subjects. Luminol-enhanced chemiluminescence depends on the formation of hypochlorous acid from H2O2 by myeloperoxidase (17), and PMN from patients with a myeloperoxidase deficiency exhibit normal generation of O2•⁻ and H2O2, but significantly depressed chemiluminescence (18,19). Thus, the difference in findings between our study and that of Lucchi et al. (7) may result from a relative deficiency, or inhibition of, myeloperoxidase in uremic PMN. We know of no data to support or refute this possibility. Vanholder et al. (10) measured the oxidative burst indirectly as 14CO2 production from glucose-1-14C over 60 min. They found reduced 14CO2 production in patients with serum creatinine concentrations of more than 6 mg/dL compared with that in normal subjects. Because of the longer measurement time, Vanholder et al. may have measured the total metabolic capacity of the PMN, rather than their ability to generate the short-lived oxidative burst. A reduced total metabolic capacity might not be unexpected in patients with far-advanced renal failure, who are frequently treated by low-protein diets and who may also have low caloric intakes. Detailed kinetic studies with both assay systems would be required to test this possibility. In the only kinetic data reported to date. Abrutyn et al. (20) found the rate of oxygen consumption during the first 10 min after the phagocytosis of opsonized zymosan to be the same in PMN from normal and uremic subjects.

Our study and the two earlier studies (7,10) also differ in the nature of the agonists used to stimulate the oxidative burst. We used a variety of agonists that stimulate the oxidative burst by direct action on protein kinase C (PKC), by interaction with a chemoattractant receptor (fMLP), and by phagocytosis (S. aureus). Lucchi et al. (7) (opsonized zymosan) and Vanholder et al. (10) (zymosan and latex particles) used only phagocytic stimuli. Moreover, Vanholder et al. relied on the plasma present in the incubation mixture to opsonize their particles. The possibility that inadequate opsonization played a role in their findings is suggested by the observations of Abrutyn et al. (20). They found no difference in 14CO2 production from glucose-1-14C between PMN from normal subjects and uremic subjects with a mean serum creatinine concentration of 7.2 mg/dL using methodology similar to that of Vanholder et al. except that opsonized zymosan was used as the agonist. These contradictory results, and the difficulty in comparing different methodologies, underscore the need for further studies to identify the specific biochemical nature of the defect(s) in uremic PMN.

The altered oxidative burst in uremic PMN is specific for plasma membrane receptor-mediated activation. The enhanced oxidative burst in uremic PMN was seen after phagocytosis, which requires immunoglobulin and complement receptors, and after stimulation with the chemoattractant fMLP. There was no difference in the oxidative burst stimulated by the direct activation of protein kinase C with PMA. These findings suggest that uremia produces an alteration in transmembrane signaling pathways at the point before the generation of intracellular messengers. Although the precise requirements for oxidative burst enzyme activation remain to be determined, there is evidence that the activation of protein kinase C by diacylglycerol produced from the phospholipase D-mediated metabolism of membrane phospholipid is a significant event (21–23). It is well known that immunoglobulin and formyl peptide receptors use different transmembrane signaling pathways to activate oxidative burst enzymes. Formyl peptide receptors are coupled to effector enzymes, such as phospholipase D and phospholipase C, by pertussis toxin-sensitive G proteins (4). Immunoglobulin receptors are not coupled to pertussis toxin-sensitive G proteins and may use tyrosine kinase activity to initiate intracellular messenger formation (24–26). Thus, the site in the transmembrane signaling cascade at which uremia alters PMN function cannot be clearly identified from our studies. Possible activities of uremia include nonspecific alterations of plasma membrane, resulting in enhanced second messenger formation, no matter what receptor is activated, alteration of multiple signaling pathways, or a single alteration at a, heretofore unidentified, common point in formyl peptide receptor and immunoglobulin receptor-initiated signaling pathways.

The inability of TNF-α to prime PMN function in our patients (Figure 2) suggests that uremic PMN exist in a primed state. Chronic renal insufficiency and exposure to TNF-α resulted in similar degrees of enhanced PMN oxidative burst that were not additive. However, these data do not establish that the mechanisms of priming by TNF-α and uremia are the same. It is possible that priming by either TNF-α or uremia results in an enhanced response that cannot be further enhanced by any other priming agent.

Our findings suggest that a reexamination of the question of the susceptibility of uremic patients to infection is in order. The primed state of uremic PMN indicates that any increase in susceptibility to infection is likely due to other alterations in the immune response to bacteria. The enhanced responsiveness of uremic PMN could play a role in the progression of renal insufficiency to chronic renal failure. There is evidence that the production of oxygen radicals within diseased kidneys results in progressive damage and loss of function (27). Primed PMN in patients with chronic renal insufficiency may make the intrarenal release of oxygen radicals more likely.

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