Neutrophil Apoptosis and Dysfunction in Uremia

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Abstract. The high prevalence of bacterial infections among patients with end-stage renal disease suggests that "professional" phagocytes such as neutrophils are functionally impaired. This dysfunction has been ascribed to uremic toxins, malnutrition, and dialysis. The aim of this study was to investigate the contribution of apoptosis to neutrophil dysfunction in uremia. Neutrophils harvested from uremic patients ($n = 6$) and age-/gender-matched healthy control subjects ($n = 6$) were incubated with either 50% autologous plasma or 10% fetal calf serum. After 24-h incubation, apoptosis was quantified by flow cytometry by using propidium iodide nuclear staining. Neutrophils from healthy volunteers were also incubated with either 50% heterologous normal or uremic plasma. After 24-h incubation, apoptosis was quantified by flow cytometry and transmission electron microscopy. In addition, superoxide production was determined by measuring the capacity to reduce ferri- to ferro-cytochrome C by using 4-β-phorbol 12-β-myristate 13-α-acetate or *N*-formyl methionyl-leucyl-phenylalanine (fMLP) for stimulus. Phagocytosis was determined by the uptake of ^{14}C-labeled heat-killed *Staphylococcus aureus*. Compared with normal neutrophils, uremic neutrophils demonstrated greater apoptosis in the presence of autologous plasma (9 ± 4 *versus* $19 \pm 6\%$, $P = 0.01$) as well as 10% fetal calf serum (19 ± 7 *versus* $31 \pm 6\%$, $P = 0.03$). Furthermore, compared with normal neutrophils exposed to heterologous normal plasma, those exposed to heterologous uremic plasma exhibited higher apoptosis rates (19 ± 3 *versus* $40 \pm 5\%$, $P = 0.002$), lower fMLP-stimulated superoxide production (22.6 ± 2.5 *versus* 15.5 ± 1.1 nmol $O_2^{\cdot-}/3.12 \times 10^5$ cells/30 min, $P = 0.01$), and a lower phagocytosis index ($38 \pm 3\%$ *versus* $27 \pm 5\%$, $P = 0.04$). Apoptosis correlated inversely with fMLP-stimulated superoxide production ($r = -0.60$, $P = 0.04$) and phagocytosis ($r = -0.57$, $P = 0.05$). These results suggest that uremic neutrophils undergo accelerated *in vitro* apoptosis. Furthermore, uremic plasma accelerates apoptosis of normal neutrophils, resulting in a dysfunctional pattern that is similar to that observed in uremia.

Infection is one of the leading causes of morbidity and mortality in patients with end-stage renal disease (ESRD) (1–4). Indeed, bacterial infections account for 15% of deaths among ESRD patients on dialysis therapy (5). The pathogenesis underlying this increased susceptibility to infection has not been clearly elucidated, but it is believed to be attributable to impaired host defense mechanisms (6). In addition to abnormalities in cell-mediated immunity involving primarily T lymphocytes, "professional" phagocytes such as neutrophils exhibit impaired chemotaxis, adherence, reactive oxygen species (ROS) production, and phagocytosis (4,6–9). Furthermore, the high frequency of bacterial infections in uremic patients suggests that neutrophils are affected by the uremic environment. The mechanisms responsible for altered neutrophil functions are only partially understood and have been loosely ascribed to uremic toxins, malnutrition, iron overload, increased intracellular ionized calcium, and dialysis therapy *per se* (6).

It has become apparent in recent years that immune responses may be regulated in part by a subtle balance between recruitment and constitutive cell death or apoptosis of immunocompetent cells. Among leukocytes, neutrophils have the shortest half-life (10), and they rapidly die *in vitro* by apoptosis (11). Furthermore, *in vivo*, mature circulating neutrophils spend approximately 12 h in the bloodstream after which time they migrate into normal tissues or are drawn by chemotactic stimuli to inflamed tissues, where they undergo apoptosis and are engulfed by tissue phagocytes. When compared with cell necrosis, apoptosis is believed to be protective for the surrounding tissues, as the cell membrane integrity is retained until the very late stages, avoiding the leakage of harmful components (12).

Recent studies suggest that in uremic patients, peripheral blood mononuclear cells, including T lymphocytes and monocytes, undergo accelerated apoptosis (13,14). However, little is known of neutrophil apoptosis in uremia, its magnitude, or its contribution to neutrophil dysfunction. Neutrophils undergoing apoptosis are dysfunctional (15). Indeed, impaired chemotaxis and phagocytosis, as well as a reduced ability to generate superoxide in response to various stimuli, have been observed in apoptotic neutrophils (15). This dysfunctional pattern is similar to that of uremic neutrophils that demonstrate altered oxidative responses and impaired chemotaxis, aggregation, and phagocytosis. Therefore, it is conceivable that uremia induces apoptosis that may be responsible in part for the neutrophil

dysfunction observed in these patients. Consequently, the aim of the present study was to investigate the contribution of apoptosis to neutrophil dysfunction in uremic patients. This was achieved by examining whether uremic neutrophils cultured *in vitro* undergo accelerated apoptosis and whether uremic plasma influences neutrophil apoptosis and functions.

Materials and Methods

Study Design

To evaluate apoptosis in uremic neutrophils and the apoptosis-inducing activity of uremic plasma, neutrophils from hemodialysis patients and matched healthy volunteers were incubated in culture medium supplemented with either autologous plasma, heterologous plasma (from the matched pair), or fetal calf serum (FCS), respectively. After the incubation period, neutrophil aliquots were processed for quantification of apoptosis.

To evaluate the impact of apoptosis induced by uremic plasma on cellular functions, neutrophils from healthy volunteers were incubated in culture medium supplemented with heterologous "normal" or "uremic" plasma, respectively. After the incubation period, neutrophil aliquots were processed for quantification of apoptosis, superoxide production, and phagocytosis.

To address the effect of heat inactivation on the apoptosis-inducing activity of uremic plasma, we ran additional experiments in which neutrophils from a healthy donor were incubated in culture medium supplemented with either unheated or heat-inactivated uremic plasma. After 24-h incubation, apoptosis was quantified by Acridine Orange/ethidium bromide dual uptake.

Subjects and Sample Collection

In the first part of the study, blood samples were collected from patients with ESRD on long-term hemodialysis (HD). Patients with acute infection or blood transfusion in the past month, chronic infections (hepatitis B, hepatitis C, HIV, osteomyelitis), active immunologic disease (systemic lupus erythematosus, rheumatoid arthritis), immunosuppressive therapy, previous transplantation, or a history of malignancy were excluded from the study. Six patients (four men, two women) with a mean age of 61 ± 7 yr were examined. These patients were dialyzed for 3.4 ± 0.3 h three times weekly, had a serum albumin of 4.1 ± 0.1 g/dl, and achieved a single pool Kt/V of 1.56 ± 0.09 (average of three values over 3 consecutive months). Six age- (mean age, 62 ± 8 yr) and gender-matched healthy subjects (serum creatinine ≤ 1.5 mg/dl) served as control subjects. Collection of blood samples was approved by the Human Investigation Review Committee, and all participants gave informed consent. In patients on HD, heparinized whole blood (10 IU/ml) was drawn from the fistula needle immediately before dialysis, and in healthy volunteers from a peripheral vein. In all instances, a 15-ml blood sample was obtained from each donor: 10 ml were used to harvest neutrophils, and plasma was separated from the remaining 5 ml. All plasma samples were filtered through a 0.8-μm cellulose acetate filter (Uniflo®-25; Schleicher & Schuell, Keene, NH) and stored at -70°C.

In the second part of the study, 60 ml of heparinized (10 IU/ml) blood samples were procured from two healthy volunteers and were used to harvest neutrophils. In addition, 5-ml blood samples were obtained from six healthy volunteers and six HD patients, respectively. Plasma was separated, filtered as described previously, and stored at -70°C.

Neutrophil Isolation

Water, cell culture media, and other solutions used in the study were subjected to ultrafiltration by using a polyamide hollow-fiber ultrafilter (U2000, Gambro AB, Hechingen, Germany), to remove cytokine-inducing agents. Neutrophils were harvested by Ficoll-Hypaque separation and hypotonic lysis of erythrocytes as described previously (16). In brief, each 10-ml sample of heparinized (10 IU/ml) blood was diluted in 20 ml of normal saline (Abbott Laboratories, Rockford, IL), underlayered with 10 ml of Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO; Nycomed, Inc., New York, NY), and centrifuged at $450 \times g$ for 40 min at room temperature. After discarding the mononuclear cell layers, Dulbecco's phosphate-buffered saline (PBS) (Life Technologies, Grand Island, NY) and 3% dextran (Sigma Chemical Co.) were added to the buffy coat. After 15 min, the leukocyte-rich supernatant was harvested and centrifuged at $450 \times g$ for 5 min at 4°C. Residual erythrocytes were subjected to hypotonic lysis. This procedure was repeated once or twice until the cell pellet appeared relatively free of erythrocytes. Cells were suspended in ice-cold PBS, and neutrophils were counted by using a standard hemocytometer. The purity of neutrophils was greater than 95%, and viability greater than 99% as judged by the trypan blue exclusion method. Neutrophils were suspended at 5×10^6/ml in ultrafiltered culture medium containing RPMI 1640 (pH 7.4, Sigma Chemical Co.), 10 mmol/L L-glutamine, 24 mmol/L NaHCO$_3$ (Mallinckrodt, Paris, KY), 10 mmol/L Hepes (Sigma Chemical Co.), 100 U/ml penicillin, and 100 μg/ml streptomycin (Irvine Scientific, Santa Ana, CA).

Cell Incubation

In the first part of the study, a 0.5-ml suspension of neutrophils (2.5×10^6 cells) was aliquoted into a 12-$\times$-75-mm polystyrene tube (Becton Dickinson Labware, Lincoln Park, NJ) and centrifuged at $450 \times g$ for 5 min at 4°C. The cells were resuspended in 1 ml of ultrafiltered culture medium supplemented with either 50% autologous plasma, 50% heterologous plasma (from the matched pair), or 10% FCS, and then incubated for 24 h at 37°C with 5% CO$_2$. After the incubation period, the cells were gently resuspended in the same medium, washed with 3 ml of PBS, and centrifuged at $450 \times g$ for 5 min at 4°C. The cell pellet was then fixed in 2 ml of 70% ethanol (Aaper Alcohol & Chemical Co., Shelbyville, KY) and incubated at 4°C for a minimum of 1 h. The cells were then processed for quantification of apoptosis.

In the second part of the study, a 0.5-ml suspension of neutrophils (2.5×10^6 cells) was aliquoted into a 12-$\times$-75-mm polystyrene tube (Becton Dickinson Labware) and centrifuged at $450 \times g$ for 5 min at 4°C. The cells were resuspended in 1 ml of ultrafiltered culture medium supplemented with 50% heterologous "normal" or "uremic" plasma, and then incubated for 24 h at 37°C with 5% CO$_2$. Each experiment was performed in duplicate. After the incubation period, neutrophil aliquots from each of the duplicated tubes were processed for quantification of apoptosis, phagocytosis (tube 1), and superoxide production (tube 2), respectively.

DNA Content Analysis by Flow Cytometry

Propidium iodide (PI) is a fluorochrome that is used in flow cytometry as a nuclear marker. As a result of the activation of an endonuclease that cleaves DNA, apoptotic cells have a low DNA content, therefore, less DNA stainability. The assessment of neutrophil apoptosis by flow cytometry was performed according to a modified version of a previously described technique (17). In brief, ethanol-fixed neutrophils were centrifuged at $450 \times g$ for 5 min,

resuspended in PBS, and stained with 50 μg/ml PI (Sigma Chemical Co.). The cells were incubated in the dark at room temperature for 15 min and then immediately subjected to flow cytometric analysis. Flow cytometric analysis was carried out at a flow rate of 1000 events/s by using a dual laser flow cytometer (EPICS® XL-MCL, Coulter Corp., Miami, FL). A total of 10,000 events were counted. Cell debris and clumps were excluded from the analysis by gating single cells in the forward and side light scatters. PI was excited by using the 488-nm ultraviolet line of the Argon laser. For each donor, neutrophils were immediately fixed after isolation, and analyzed first, to determine the gates delineating the hypodiploid cell population with low PI uptake. Neutrophils with low PI uptake were considered apoptotic. Acquired data were analyzed with PC-based software (WinMDI version 2.6, The Scripp Research Institute, La Jolla, CA). The proportion of apoptotic neutrophils was defined as the number of apoptotic cells divided by the total number of cells $\times$ 100.

Superoxide Production

The production of superoxide (O_2^-) by neutrophils was determined by measuring the capacity of cells to reduce ferri- to ferro-cytochrome C (16). In brief, after 24-h incubation, neutrophils were washed in PBS and resuspended in 1 ml of Hanks' balanced salt solution (HBSS) (2.5×10^6 cells/ml). Neutrophil aliquots of 125 μl (3.12×10^5 cells) were incubated in Eppendorf™ tubes (USA/Scientific Plastic, Ocala, FL) in the presence of 25 μl of ferri-cytochrome C (12.3 mg/ml) (Sigma Chemical Co.), 25 μl of stimuli—10 nM solution of 4-β-phorbol 12-β-myristate 13-α-acetate (PMA; Sigma Chemical Co.) or 10 μM solution of N-formyl methionyl-leucyl-phenylalanine (fMLP; Sigma Chemical Co.)—with or without 25 μl of superoxide dismutase (SOD) (1 mg/ml) (Sigma Chemical Co.). HBSS was added to finalize the volume at 250 μl. After a 30-min incubation at 37°C with rotational agitation, the tubes were placed on ice and centrifuged at 14,000 rpm for 1 min at 4°C. All experiments were performed in duplicate. The amount of O_2^- produced was quantified by measuring the change in spectrophotometric absorbance of the cell-free supernatant at dual wavelength (550-nm test filter with a reference wavelength of 570 nm), by using a microplate reader (MRX-500; Dynatech Laboratories, Chantilly, VA). The results are expressed as nanomoles of O_2^- produced/3.12×10^5 cells per 30 min, based on an extinction coefficient for a 1-cm light path (ferro $-$ ferri) of 21.1.

Phagocytosis

Phagocytosis of opsonized bacterial particles was determined by measuring the uptake of ^{14}C-labeled heat-killed *Staphylococcus aureus* as described previously (16). In brief, after 24-h incubation, neutrophils were washed in PBS and resuspended in 800 μl of HBSS (2.5×10^6 cells) in a 12-$\times$-75-mm polypropylene tube (Becton Dickinson Labware), in the presence of 100 μl of 10% autologous serum and 100 μl of ^{14}C-labeled bacteria (5×10^7/ml). The cells were incubated for 30 min at 37°C with rotational agitation. At the end of the incubation period, phagocytosis was stopped by adding 2 ml of ice-cold PBS, and the cells were centrifuged at 450 $\times$ *g* for 5 min at 4°C. The supernatant was aspirated to remove non–cell-associated bacteria. Cells were resuspended in 2 ml of PBS, and the wash procedure was repeated twice. After the final wash, 500 μl of 0.2N sodium hydroxide was added, and the cells were incubated overnight at 37°C. At the end of the incubation period, 200 μl of 3% acetic acid and 300 μl of HBSS were added. The 1-ml cell suspension was mixed with 9 ml of scintillation solution Ecoscint™ H (National Diagnostics, Atlanta, GA), and cell-associated counts were determined by a 1219 Rackbeta liquid scintillation counter (LK 66 Wallac, Turku, Finland).

Phagocytosis was expressed as an index (ratio of cell-associated counts/total counts added $\times$ 100).

Transmission Electron Microscopy

To validate the phenotypical findings observed by flow cytometry, transmission electron microscopy (TEM) was also performed as described previously (18). In brief, neutrophil suspensions obtained from a healthy volunteer were incubated in ultrafiltered culture medium supplemented with 50% heterologous normal plasma $\pm$ 10 μg/ml cycloheximide (Aldrich Chemical, Milwaukee, WI) or 50% heterologous uremic plasma, for 24 h at 37°C with 5% CO_2. After the incubation period, cells were washed twice in PBS, pelleted at 250 $\times$ g for 5 min, and fixed with 2.5% glutaraldehyde at 37°C for 2 h. Cells were then washed with 0.1 M sodium cacodylate and post-fixed with 1% osmium tetroxide for 1 h at 0°C. Subsequently, cells were washed and resuspended several times with increasing concentrations of ethanol (30 to 100%). After ethanol treatment, cells were embedded in polybed 812 resins (Polysciences, Warrington, PA) with *en bloc* stain of 1.5% uranyl acetate in 50% ethanol. Gold thickness sections were cut with a diamond knife on a Reichert Ultracut E ultra-microtome. The sections were poststained with Sato's lead stain and 3% uranyl acetate. The sections were finally examined and photographed by an independent observer, under a Phillips CM-10 electron microscope (Phillips, Eindhoven, The Netherlands). For each sample, two microscopic fields were photographed and apoptosis counts were performed by two readers blind to the protocol.

Acridine Orange/Ethidium Bromide Uptake

This method of detecting apoptosis is based on the loss of plasma membrane integrity as cells die (19). In brief, Acridine Orange (100 μg/ml; Sigma Chemical Co.) and ethidium bromide (100 μg/ml; Sigma Chemical Co.) stock solutions were prepared in PBS, respectively. A dye cocktail was prepared by adding equal volumes of ethidium bromide and Acridine Orange. Five microliters of cocktail was added to 125 μl of cell suspension, mixed gently, and allowed to stand at room temperature for approximately 1 to 2 min. Ten microliters of cell suspension was placed on a graded glass slide (Gurley Precision Instruments, Fisher Scientific, Pittsburgh, PA), covered with a coverslip, and examined under $\times$20 magnification with a fluorescence microscope (Labophot, Nikon, Japan). Counts were performed by a reader blind to the protocol. Cells were identified as viable (bright green nucleus with intact membrane), early apoptotic (bright green nucleus showing condensation of chromatin as dense green areas in the nucleus), late apoptotic (orange nucleus showing condensation of chromatin as dense orange areas), and necrotic (orange nucleus with intact structure).

Statistical Analyses

Statistical analysis was performed by using the True-Epistat software package (Epistat Σ Services, Richardson, TX). Continuous variables are expressed as mean $\pm$ SEM. For the quantification of apoptosis by TEM, results are presented as the mean of two pictures read by two readers blind to the protocol. t test (paired and unpaired) was used to test the significance of differences. Linear regression analysis (Pearson's coefficient) was used to assess the relationship between apoptosis and neutrophil dysfunction. Differences were considered statistically significant if $P < 0.05$.

Results

Increased Apoptosis of Uremic Neutrophils and Effect of Uremic Plasma on Apoptosis of Normal Neutrophils

After 24-h incubation with autologous plasma, apoptosis was observed in $9 \pm 4\%$ of neutrophils from healthy control subjects. In contrast, in the presence of autologous plasma, apoptosis was observed in $19 \pm 6\%$ of neutrophils from uremic patients ($P = 0.01$) (Figure 1). Likewise, after 24-h incubation with 10% FCS, apoptosis was observed in $19 \pm 7\%$ of neutrophils from healthy control subjects compared with $31 \pm 6\%$ of neutrophils from uremic patients ($P = 0.03$) (Figure 1).

Furthermore, apoptosis rates in normal neutrophils incubated with autologous plasma increased by $33 \pm 8\%$ when cells were exposed to heterologous uremic plasma ($P = 0.04$). Conversely, apoptosis rates in uremic neutrophils exposed to autologous plasma ($19 \pm 6\%$) were not significantly different from those incubated with heterologous normal plasma ($18 \pm 4\%$, $P = 0.50$). These results suggest that uremic neutrophils may be constitutively more prone to apoptosis, and substitution of uremic with normal plasma does not reduce the proportion of cells undergoing apoptosis.

Effect of Uremic Plasma on Apoptosis and Function of Normal Neutrophils

Neutrophils from healthy volunteers incubated with heterologous uremic plasma were next used as a model to study the effect of uremic plasma on apoptosis and functions of normal neutrophils. Compared with neutrophils from healthy volunteers exposed to heterologous normal plasma, those exposed to heterologous uremic plasma exhibited higher apoptosis rates ($40 \pm 5\%$ *versus* $19 \pm 3\%$, $P = 0.002$) (Figure 2), lower fMLP-stimulated superoxide production (15.5 ± 1.1 *versus*

Figure 2. Apoptosis of normal neutrophils after 24-h incubation in culture medium supplemented with either 50% heterologous normal or uremic plasma ($n = 6$). *$P = 0.002$ *versus* heterologous normal plasma.

22.6 ± 2.5 nmol $O_2^-/3.12 \times 10^5$ cells/30 min, $P = 0.01$) (Figure 3) and a lower phagocytosis index (27 ± 5 *versus* $38 \pm 3\%$, $P = 0.04$) (Figure 3). However, there was no significant difference in PMA-stimulated superoxide production between the two groups (21.1 ± 4.1 for normal plasma *versus* 24.4 ± 3.7 nmol $O_2^-/3.12 \times 10^5$ cells/30 min for uremic plasma, $P = 0.28$).

There was an inverse correlation between neutrophil apoptosis and fMLP-stimulated superoxide production ($r = -0.60$, $P = 0.04$) (Figure 4A) and phagocytosis index ($r =$
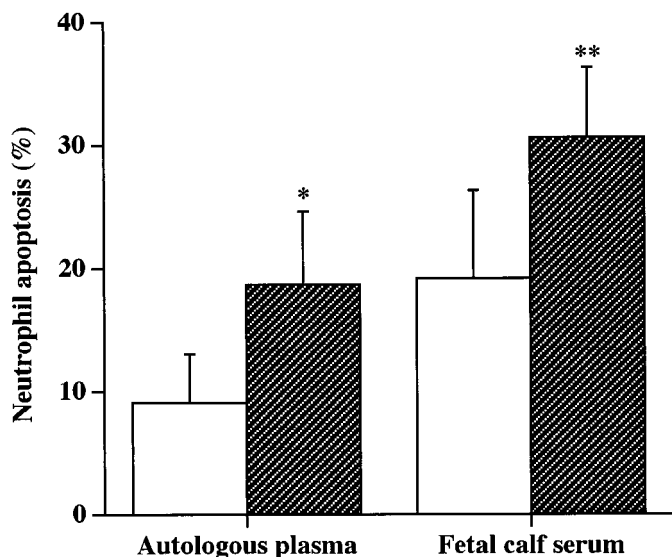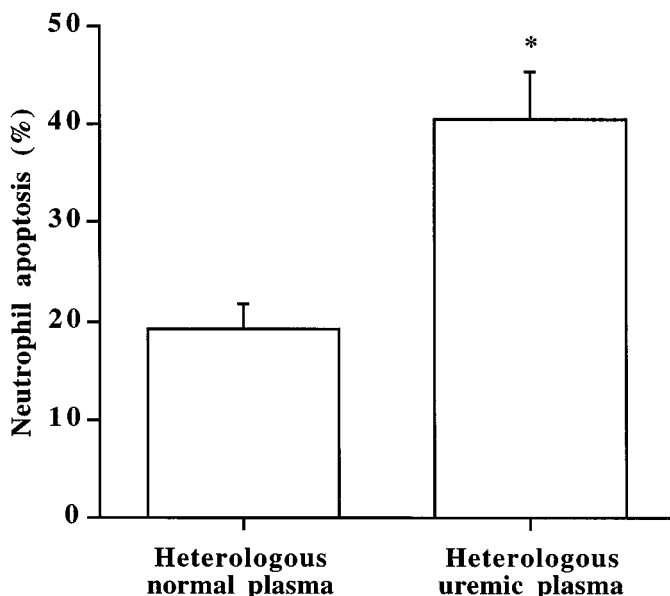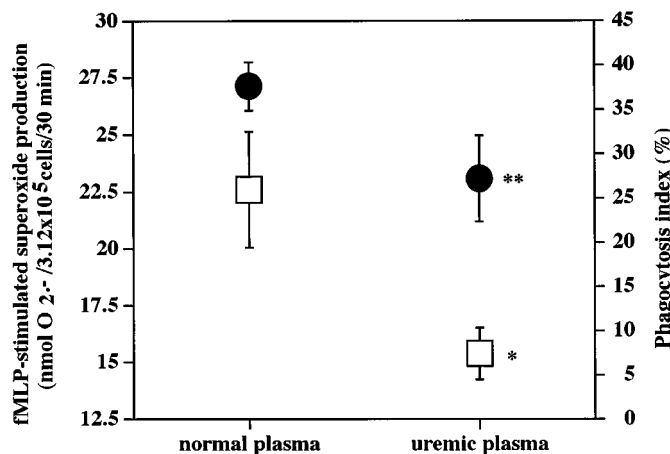
Figure 1. Apoptosis of normal and uremic neutrophils after 24-h incubation in culture medium supplemented with either 50% autologous plasma or 10% fetal calf serum ($n = 6$). *$P = 0.01$ *versus* normal neutrophils; **$P = 0.03$ *versus* normal neutrophils. □, normal neutrophils; ▨, uremic neutrophils.

Figure 3. N-Formyl methionyl-leucyl-phenylalanine (fMLP)-stimulated superoxide production ($n = 6$; □, mean) (*$P = 0.01$ *versus* normal plasma) and phagocytosis of heat-killed ^{14}C-labeled *Staphylococcus aureus* ($n = 6$; ●, mean) (**$P = 0.04$ *versus* normal plasma) by normal neutrophils after 24-h incubation in culture medium supplemented with either 50% heterologous normal or uremic plasma.
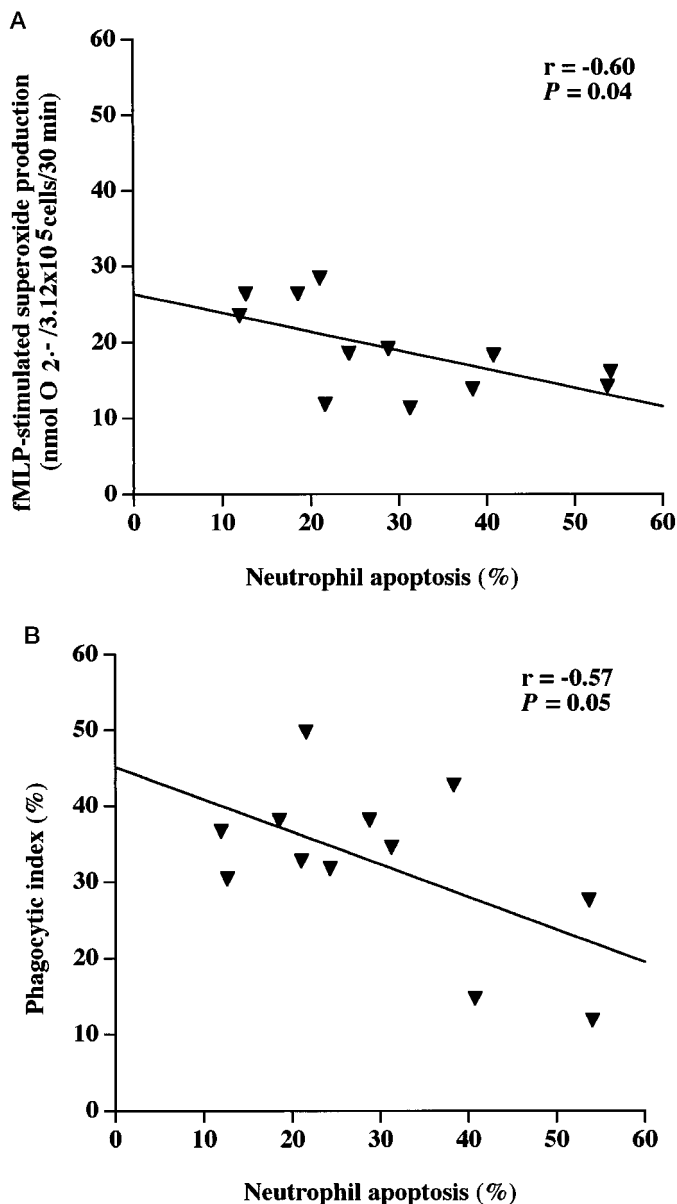
Figure 4. Correlation between neutrophil apoptosis and functional assays. Correlation between apoptosis of normal neutrophils and fMLP-stimulated superoxide production (A) and phagocytosis index (B).

-0.57, $P = 0.05$) (Figure 4B). There was no significant correlation between neutrophil apoptosis and PMA-stimulated superoxide production ($r = 0.34$, $P = 0.28$).

Transmission Electron Microscopy

To validate the phenotypic findings observed by flow cytometry, TEM was performed on normal neutrophils after 24-h incubation in the presence of heterologous normal plasma with or without cycloheximide, or uremic plasma. Quantification of apoptosis by TEM demonstrated that neutrophils incubated with heterologous normal plasma exhibited $18 \pm 1\%$ apoptosis ($n = 4$). In contrast, the proportion of apoptosis was significantly higher among neutrophils exposed to uremic plasma

($32 \pm 4\%$, $P = 0.04$, $n = 4$) or normal plasma with cycloheximide ($66 \pm 5\%$, $P = 0.004$, $n = 4$). Figure 5 illustrates a concomitant flow cytometric histogram and TEM slide of one such experiment. As seen on the histograms, compared with cells incubated with normal plasma (Figure 5A), there was a marked increase in neutrophil apoptosis in the presence of uremic plasma (Figure 5B) or normal plasma containing cycloheximide (Figure 5C). Furthermore, the typical features of apoptosis were seen on TEM, consisting of chromatin condensation, loss of cytoplasmic processes, round cell shape, and in some cells, fading of the nuclear membrane.

Effect of Heat Inactivation on Apoptosis-Inducing Activity of Uremic Plasma

To assess the effect of heat inactivation of uremic plasma on apoptosis, neutrophils isolated from a healthy volunteer were incubated for 24 h with either heterologous normal plasma ($n = 3$), uremic plasma ($n = 3$), or heat-inactivated ($56°C$ for 30 min) uremic plasma ($n = 3$). The results shown in Figure 6 suggest that, although uremic plasma accelerated *in vitro* apoptosis, heat inactivation did not abrogate its apoptosis-inducing activity.

Discussion

The increased incidence of bacterial infections among patients with renal failure (1–4) suggests that "professional" phagocytes such as neutrophils may be defective. Indeed, the functional impairment of neutrophils in uremia may play a major role in the increased susceptibility to infections. However, the pathogenic mechanisms responsible for this dysfunction are not fully understood. In this study, we observed that neutrophils isolated from uremic patients and incubated *in vitro* with autologous plasma exhibited a higher proportion of apoptosis than neutrophils isolated from healthy donors. This difference was maintained when cells from both uremic patients and healthy donors were incubated with FCS. Constitutive cellular factors, at least in part, may be responsible for this accelerated apoptosis, as neutrophils from uremic patients incubated with normal plasma had similar apoptosis rates compared with those incubated with uremic plasma. In addition, normal neutrophils incubated with uremic plasma had higher rates of apoptosis than cells exposed to autologous or heterologous normal plasma, suggesting that some putative soluble factor(s) may also induce apoptosis. Furthermore, phagocytosis and fMLP- (but not PMA-) stimulated superoxide production correlated inversely with neutrophil apoptosis, suggesting that the dysfunction of neutrophils exposed to uremic plasma is, at least in part, due to apoptosis.

Our observations are in accordance with recent studies that have shown that peripheral blood lymphocytes and monocytes obtained from uremic patients undergo accelerated apoptosis when cultured *in vitro* (13). Indeed, Matsumoto and colleagues have observed increased apoptosis of T lymphocytes from both dialyzed and undialyzed uremic patients (13). Furthermore, *in vivo*, uremic T lymphocytes expressed Fas with higher intensity than control T cells, suggesting that T lymphocyte apo-
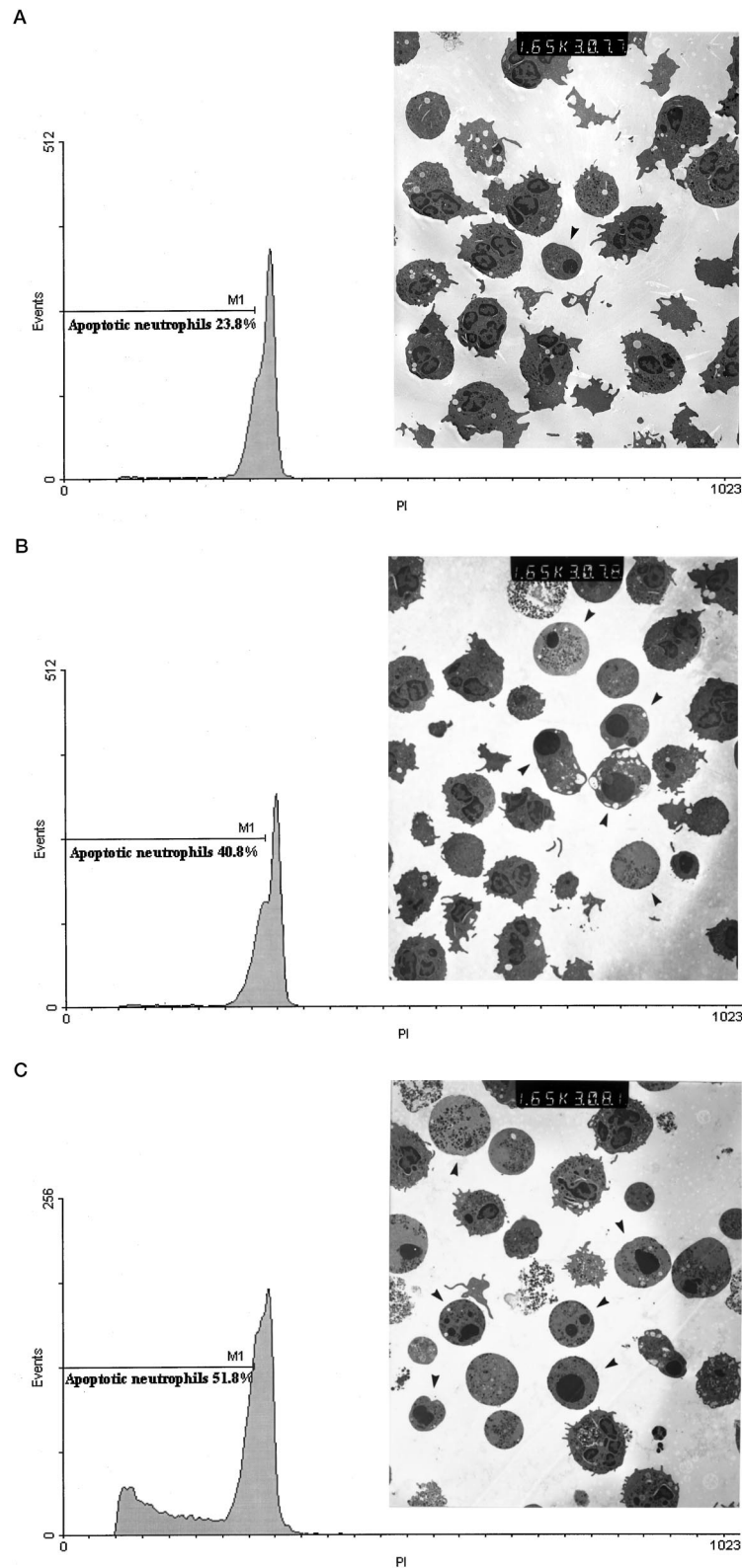
Figure 5. DNA fluorescence histograms and transmission electron microscopy (TEM) photomicrographs of normal neutrophils after 24-h incubation in culture medium supplemented with either normal plasma (A), uremic plasma (B), or normal plasma containing cycloheximide (C). Each panel represents a histogram illustrating a DNA fluorescence histogram after 24-h incubation (gray area). The gated (M1) cells in the pre-G_0 area constitute the hypodiploid apoptotic cell population. On TEM, Panel A displays only one neutrophil with the typical features of apoptosis (arrows), whereas Panels B and C have more apoptotic cells (chromatin condensation, loss of cytoplasmic processes, and round cell shape).
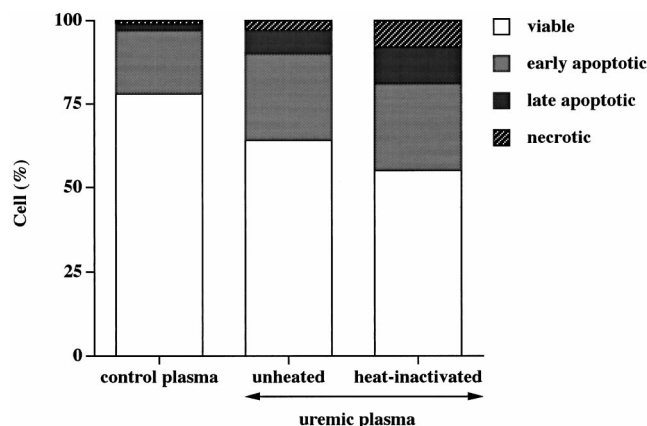
Figure 6. Apoptosis and necrosis of normal neutrophils after incubation with either heterologous normal plasma (*n* = 3), uremic plasma (*n* = 3), or heat-inactivated uremic plasma (*n* = 3), by using dual staining with Acridine Orange and ethidium bromide.

ptosis may be mediated by the Fas system. When a comparison was made between two uremic groups, Fas intensity on T cells was significantly higher in undialyzed than in hemodialyzed patients (13). The authors concluded that uremic T cells undergo apoptosis by the Fas system, which may be partly inhibited by HD therapy. Heidenreich and colleagues have observed enhanced apoptosis of uremic monocytes *in vitro* as well. This enhancement was accompanied by a decrease in tumor necrosis factor-α production, and supplementation of monocyte cultures with exogenous tumor necrosis factor-α decreased apoptosis rates, suggesting that proinflammatory mediators may modulate the survival of senescent monocytes (14). In addition, a reduced ability of monocytes to inhibit *Candida albicans* growth correlated inversely with DNA fragmentation, suggesting that senescent monocytes are functionally impaired (14).

The impact of apoptosis on neutrophil functions has been reported in normal cells. Whyte and colleagues evaluated apoptosis and function of senescent normal neutrophils, and demonstrated a reduced ability to spread, change shape, migrate in response to chemotactic agents, and to degranulate (15). In addition, phagocytosis and fMLP-stimulated superoxide production were particularly depressed in apoptotic cells, whereas PMA-stimulated superoxide production was preserved (15). The preservation of a response to PMA, a cell receptor-independent stimulus, suggests that some intracellular signaling pathways relevant to superoxide production may be retained in apoptotic neutrophils.

The low rate of neutrophil apoptosis that we detected differs from that observed by Whyte and colleagues (15). This discrepancy may be due to several factors, including differences in tissue culture medium, concentration of autologous plasma, and isolation techniques that may have been associated with neutrophil activation.

Studies assessing the respiratory burst of uremic neutrophils are numerous but have yielded conflicting results (7,20–22). Lewis and colleagues have shown that fMLP- (but not PMA-)

stimulated superoxide production is depressed in uremic neutrophils harvested from dialysis patients (7). These results are in accordance with our findings, and mirror the functional impairments that are commonly seen in neutrophils undergoing apoptosis (15). In contrast, other studies suggest that oxidative burst is enhanced in uremia (20–22). Indeed, Ward and McLeish have demonstrated that neutrophils harvested from undialyzed uremic patients with various degrees of chronic renal insufficiency have an enhanced hydrogen peroxide and superoxide production in response to receptor-mediated stimuli (fMLP, *Staphylococcus aureus*), but not to a receptor-independent stimulus (PMA) (22). Furthermore, a recent study suggests that fMLP-stimulated neutrophils undergo accelerated apoptosis by a superoxide release-dependent pathway (23). Consequently, it is conceivable that at an early phase, uremia enhances neutrophil oxidative burst, which, in turn, induces apoptosis, resulting at a later phase, in functional impairment.

Although neutrophil dysfunction was observed *in vitro* under cultured conditions, it remains to be determined whether *in vivo*, apoptosis accounts for uremic dysfunction of neutrophils, and to what extent. This work is currently in progress. Nevertheless, the apoptosis-inducing activity of uremic plasma that we observed suggests a role for soluble factor(s). In recent years, a number of uremic toxins that affect neutrophil functions have been identified. These include parathyroid hormone (24), *p*-cresol (25), spermine (26), and a series of granulocyte inhibitory proteins, GIP-I (with homology to Ig light chains) (27), GIP-II (with homology to β2-microglobulin) (28), angiogenin (29), complement factor D (30), and chemotaxis inhibiting protein (with partial homology to ubiquitin) (6). However, none of these molecules had been examined with respect to its apoptosis-inducing potential. Therefore, further studies on the effect of these uremic toxins on neutrophil apoptosis are needed.

Although our results suggest that heat inactivation of uremic plasma does not abrogate its apoptosis-inducing activity, it appeared to have resulted in increased cell necrosis (Figure 6). We can only speculate whether heat inactivation resulted in cell deprivation from putative growth factor(s), resulting in their demise.

In summary, we demonstrated that apoptosis is accelerated in uremic neutrophils. Furthermore, uremic plasma induces apoptosis in normal neutrophils, resulting in a dysfunctional pattern that is similar to that observed in uremic neutrophils. We propose that both constitutive cellular as well as soluble plasma factors are responsible for this accelerated apoptosis, which may lead to cell dysfunction.

Acknowledgments

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