A Defect in the Oxidative Metabolism of Human Polymorphonuclear Leukocytes That Remain in Circulation Early in Hemodialysis

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Human granulocytes harvested from uremic volunteers 15 min after the initiation of dialysis (at the nadir of neutropenia) were compared to predialysis controls. These intradialysis cells had a significant defect in peak luminol-enhanced chemiluminescence in response to opsonized zymosan, f-Met-Leu-Phe, and phorbol myristate acetate relative to predialysis control cells from the same patients. This defect could not be explained by a decrease in PMN myeloperoxidase concentration. H2O2 secretion by intradialysis cells (2 patients) was also depressed relative to predialysis controls. The ability to perform an independent function, orientation (polarization), was normal in both predialysis and intradialysis cells relative to control. Whereas 125I-labeled formyl peptide binding studies demonstrated identical values for affinity and receptor number for predialysis and normal control cells, intradialysis cells displayed a 27% decrease in receptor number. This decrease in available receptors may be related to the decreased chemiluminescence observed in response to f-Met-Leu-Phe. Furthermore, the results are consistent with the hypothesis that a defective PMN population remains in the circulation during the neutropenia of hemodialysis.

IN THE ABSENCE of infection or inflammation, only mature polymorphonuclear neutrophils (PMNs) with a homogeneous morphological appearance can be found in circulation. However, several lines of evidence suggest that subpopulations of mature PMNs may exist. Klempner and Gallin found that only 80% of circulating PMNs were able to form (Fc) rosettes with IgG-coated sheep red blood cells. Furthermore, cells that do not rosette have been reported to be defective in a variety of functional assays, including their ability to ingest and kill S. aureus. More recently, Seligman and his colleagues reported a heterogeneity in the depolarization of normal PMNs in response to the soluble chemotactic peptide f-Met-Leu-Phe.

Shortly after the initiation of hemodialysis or cardiopulmonary bypass, a profound leukopenia is observed. This phenomenon is believed to result from the aggregation of PMNs in the pulmonary vascular tree in response to one or more complement fragments. A previous study of the PMNs that remain in circulation (intradialysis cells) has revealed a dramatic decrease in the number of cells competent to form Fc rosettes, and a defect in the ability of this population to aggregate in response to C5a des arg. It has been suggested that this "defective" subpopulation may surface in a wide variety of clinical disorders characterized by the in vivo activation of complement, and that this population might then contribute to the inception of infection or hinder a normal recovery from infection.

The ability of granulocytes to reduce molecular oxygen to superoxide, hydrogen peroxide, and perhaps other oxidizing agents, is crucial to the bactericidal process. In order to better define PMNs that remain in circulation during dialysis, we investigated their oxidative metabolism using luminol-enhanced chemiluminescence. In this article we report a significant defect in chemiluminescence among intradialysis cells in response to several different stimuli. Our results suggest that this abnormality may in part be explained by altered membrane surface characteristics that lead to reduced binding of stimulus to this population of PMNs.

MATERIALS AND METHODS

Patient Population

Sixteen adults with chronic renal failure undergoing hemodialysis were entered into this study after providing informed consent according to the guidelines of the Human Research Committee of the University of North Carolina. All patients were dialyzed with a single use Cordis-Dow 1.8 D filter (Cordis-Dow Corporation, Miami, Fla.). Blood samples were obtained from the afferent (patient to dialyzer line) immediately before initiation of dialysis and 15-20 min later. On selected patients, automated leukocyte and 10,000 differential cell counts were determined by the Hemalog D system (Technicon Instrument Corp., Farriyown, N.Y.) on 1 cc of EDTA-anticoagulated blood.

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Granulocyte Preparation

Purified preparations of polymorphonuclear leukocytes were prepared as previously described by plasmagel (Roger Bellon Laboratories, Neuilly, France) or 3% dextran sedimentation (Pharmacia, Piscataway, N.J.), followed by Ficoll-Hypaque gradient centrifugation. Red blood cells were removed by hypotonic lysis. All preparations contained >95% PMNs. Cells were suspended in either Hank’s (HBSS) or Gey’s (GBSS) balanced salt solutions (GIBCO, Grand Island, N.Y.).

Preparation of Stimuli

A variety of stimuli capable of the initiation of PMN oxidative metabolism were employed. Zymosan (Sigma Chemical Co., St. Louis, Mo.) was boiled for 60 min, washed 3 times, suspended in GBSS, and frozen at -70°C until use. Zymosan was opsonized the week of employment in pooled human serum and used at a concentration of 1 mg/ml. The chemotactic peptide n-formyl-methionyl-leucyl-phenylalanine (f-Met-Leu-Phe) was provided by Dr. Ralph Snyderman, Duke University, and was suspended in GBSS and used at a concentration of 10⁻¹ M unless otherwise indicated. Phorbol myristate acetate (Midland Chemical Co., Brewster, N.Y.) was frozen in dimethyl sulfoxide (1 mg/ml) at -70°C, and diluted to a final concentration of 1 μg/ml in GBSS for use. Endotoxin-activated serum was prepared as previously described.

Chemiluminescence

Luminol-enhanced chemiluminescence was performed in a Packard luminometer. Two-hundred-microliter (1.2-2.5 x 10⁵ PMNs/ml) samples were suspended in GBSS with 5 X 10⁻² M 5-amino-2,3 dihydro-1,4 phthalizidine (Luminol, Sigma Chemical Co.) and 0.01% bovine serum albumin. Determination of baseline light emission was made before addition of a stimulus. Light emission was subsequently recorded each minute for 10-sec intervals until peak emission was detected both in the presence and absence of a stimulating agent. In some experiments, luminescence was measured until return to a baseline value. Cell-free controls did not yield significant light emission.

H₂O₂ Secretion

H₂O₂ secretion was determined based on the oxidation of scopoletin in the presence of horseradish peroxidase as described by Root et al. For these experiments, the secretion of H₂O₂ by PMNs in response to 50 ng/ml PMA of over a 5-min period was determined.

Assessment of Cellular Myeloperoxidase Concentration

An estimation of myeloperoxidase concentration was obtained in selected patients by noting the deflection of light in a polaroid photograph of the peroxidase channel of an automated cytochemical analyzer (Hemalog D, Technicon, Tarrytown, N.Y.). The deflection was converted to volts to allow quantitation. This instrument has been used to identify myeloperoxidase-deficient patients and to quantify degranulation.

Polarization (Orientation) of PMNs

Polarization of PMNs in response to a chemical gradient is the initial component of the chemotactic response. For this assay, PMNs were suspended in HBSS containing 0.01 M HEPES, pH 7.2. Cells (5 x 10⁵, 0.5 ml vol) were prewarmed for 5 min at 37°C prior to the addition of an appropriate stimulus and incubated for 10 min; the reaction was terminated by the addition of 1 ml ice-cold 10% formalin, pH 7.2. Cells were fixed for 30 min at 4°C, centrifuged, and resuspended in 0.2 ml H₂O. The number of cells that developed a characteristic polarized appearance among 200 counted was scored by phase microscopy; all assays were done in duplicate.

Radioiodine Formyl Peptide Binding Assay

N-formyl-Nle-Leu-Phe-Nle-Tyr-Lys (formyl peptide) used in this assay was synthesized by S. Wilkinson (Wellcome Research Laboratories, Beckenham, England) and was radiiodinated by a modification of the method of Hunter and Greenwood. The formyl ¹²⁵I-peptide binding assay was a modification of the method described elsewhere. Approximately 2 x 10⁵ cells and the designated concentration of formyl ¹²⁵I-peptide were incubated at 4°C for 1 hr in 200 μl of assay buffer (15 mM sodium phosphate/123 mM NaCl/0.1% bovine serum albumin, pH 6.75). The incubation was terminated by vortex mixing with 2.0 ml of cold PBS including 0.1% BSA. Samples were filtered through Whatman GFC filters, washed with 10 ml of cold PBS, and the ¹²⁵I retained by the filter was assayed directly with an efficiency of 75%. Nonsaturable binding was determined in an identical fashion with 2 x 10⁻⁵ M unlabeled formyl peptide included during the 1-hr binding incubation. Data points were routinely taken in triplicate.

Statistics

The Wilcoxon sign rank test and Kruskal-Wallis analysis of variance tests were used for statistical computation.

RESULTS

Effects of Dialysis on Peripheral Granulocyte Count

As has previously been reported, hemodialysis results in an acute, profound neutropenia. In 9 patients examined concomitantly, neutrophil count was reduced from 3810 ± 2170 cells/cu mm to 587 ± 237 cells/cu mm (mean ± SD, n = 9); leukopenia was observed in all patients subsequently studied. Leukopenia resolved within an hour of hemodialysis, with a marked increase in band count. The kinetics of dialysis-induced leukopenia employing the Hemalog D have been described in greater detail elsewhere.

Granulocyte Chemiluminescence

Because of the wide variation in absolute light emission observed among different donors (unpublished data), results obtained with intradialysis cells are expressed as a percent of the light emission of predialysis neutrophils separated under identical conditions. All pooled data refer to the determination of peak light emission.

Granulocytes harvested 15 min after hemodialysis was initiated (intradialysis cells) manifested a 38.2% ± 9.82% (mean ± SEM, n = 5, range 11%-62%) depression in light emission in response to opsonized zymosan. The kinetics of light emission by these cells are shown in Fig. 1.

Because zymosan interacts with PMNs by virtue of serum-derived opsonins, and intradialysis cells have been reported to have a decreased ability to form Fc rosettes, we reasoned that decreased Fc-mediated
binding of zymosan might account for these results. Two approaches were undertaken to exclude this possibility. First, zymosan opsonized with heat-inactivated serum was compared to control (pooled serum) in its ability to enhance chemiluminescence. Light emission by both pre- and intradialysis cells was decreased by 75% ± 4.0% and 72% ± 6.4% (mean ± SEM, n = 3), respectively, when heat-inactivated serum was used as an opsonin, indicating that heat-labile (complement) components are primarily responsible for the cellular association of zymosan in this setting. No significant difference in the stimulation of pre- or intradialysis cells was noted by zymosan opsonized with heat-inactivated serum.

Second, a variety of other stimuli were employed. As shown in Fig. 2, chemiluminescence was quantified in response to f-Met-Leu-Phe and PMA, soluble stimuli that are known to bind to unique cellular receptors.20,21,23 With each stimulus employed, light emission was significantly depressed from predialysis control (range 21%–46.8%, Fig. 2); the degree of depression observed was similar whether zymosan, f-Met-Leu-Phe, or PMA was used as a stimulus (p > 0.25, Kruskal-Wallis test).

The defect observed in light emission with opsonized zymosan and f-Met-Leu-Phe was reflected by a decrease in the peak number of counts recorded, but the peak light emission occurred at approximately the same point in time. In response to PMA, however, the latency to peak light emission was generally prolonged (data not shown). No difference in baseline light emission between predialysis (2055 ± 1046 cpm, n = 38) or intradialysis (1777 ± 1108 cpm, n = 38) PMNs was observed. In three patients, unstimulated (resting) light emission was evaluated. Although a small peak developed in the absence of a stimulus (see Fig. 1), perhaps related to adherence to glass, no significant difference between pre- and intradialysis values was observed; this peak could not be attributed to luminol alone. Because of the wide variation in light emission by PMNs among donors, it was necessary to show that difference in peak luminescence among intradialysis cells was significant (sign-rank test) as indicated by p value.

**Myeloperoxidase Concentration of Granulocytes**

Emission of light by granulocytes is related to the formation of one or more oxygen reduction products.10,11,24,25,34 Myeloperoxidase is critical to luminol-enhanced chemiluminescence.25 Depressed chemiluminescence has been reported among patients with total peroxidase deficiency,26 and we have recently confirmed this observation in our laboratory.25 To eliminate the possibility that exocytosis of primary (peroxidase-containing) granules accounted for the depressed
chemiluminescence observed, we measured the peroxidase concentration of the neutrophil population before and during hemodialysis. Because so few intradialysis cells could be recovered, conventional peroxidase determination was untenable. As an alternative, the visual display of an automated cytochemical counter was used (see Materials and Methods). In this setting, the distance of the PMN cluster from the origin (recorded in volts) is proportional to MPO concentration. The mean peroxidase concentration of the predialysis cells was 3.4 ± 0.3 V (mean ± SEM) compared to 3.3 ± 0.6 V in the intradialysis cells. This difference was not significant. The results of a related study have been published in more detail elsewhere.

H$_2$O$_2$ Secretion

To further validate the defect in chemiluminescence observed, neutrophil H$_2$O$_2$ secretion was measured. As shown in Table 1, total H$_2$O$_2$ release by intradialysis cells in response to PMA was decreased 24%-30% relative to predialysis cells. The secretion of H$_2$O$_2$ by predialysis cells was slightly greater than normal control.

Characteristics of Formyl Peptide Receptor

The specific binding of the formyl $^{125}$I-peptide to predialysis and intradialysis granulocytes was saturable at 4°C (Fig. 3). By Scatchard analysis, at saturation, the predialysis cells bound 19.5 fmole of formyl peptide per $10^8$ cells, with $K_D = 1.9$ nM. These values are indistinguishable from those obtained with cells from normal controls (data not shown). The intradialysis granulocytes obtained approximately 15 min after initiation of dialysis bound 14.0 fmole of formyl peptide per $10^8$ cells, with a $K_D = 1.75$ nM. This decrease in maximal binding represents a change in surface receptor number from approximately $115 \times 10^3$/cell to $84 \times 10^3$/cell, a decrease of 27%.

PMN Polarization (Orientation)

The previous studies suggested that the depression in PMN oxidative metabolism might, in fact, result from a decrease in receptor number, at least with respect to f-Met-Leu-Phe. To evaluate the ability of these cells to perform another function, and to further assess the degree of “resting” stimulation among intradialysis cells, we studied the ability of the PMNs to polarize in response to chemoattractants. Polarization appears to be the initial morphological alteration of leukocytes that may subsequently chemotax. As shown in Table 1, intradialysis cells were able to polarize as well as normal and predialysis cells in response to f-Met-Leu-Phe and endotoxin-activated serum. Neither pre- nor intradialysis cells were discovered to be polarized at rest (Table 2).

**DISCUSSION**

Circulating neutrophilic leukocytes include band forms and mature cells, both of which are identified by morphological criteria; in the absence of inflammation, band forms represent only a small percentage of these cells. It has recently been suggested that mature polymorphonuclear neutrophils include one or more subpopulation(s) that are inferior in their ability to form Fe rosettes, to respond to chemotactic stimuli, to adhere to nylon wool fibers, to ingest and kill staphylococci, to suppress hematopoietic activity, or to exhibit membrane depolarization in response to f-Met-Leu-Phe.
These observations may have special relevance among patients who undergo hemodialysis and cardiopulmonary bypass. Several groups of investigators have noted that shortly after the initiation of these procedures, acute leukopenia develops and is followed within 1 hr by rebound leukocytosis and an increase in band neutrophils. These changes are most probably secondary to the activation of one or more complement components, which leads to the sequestration of peripheral circulation. The magnitude of change observed is to some extent correlated with the hemodialysis coil employed. Our results among patients dialyzed on a Cordis-Dow 1.8 D coil are virtually identical to those previously reported.

It has been difficult to investigate PMN function during dialysis because only a limited volume of blood can be obtained from patients with renal failure. By using a group of nonuremic patients undergoing hemodialysis as a possible therapy for schizophrenia, Klempner and his colleagues were able to obtain a volume of leukocytes adequate for detailed study. Their results strongly suggest that the subpopulation they had previously reported predominates in the peripheral circulation 15–20 min after the initiation of hemodialysis. It was hypothesized that these cells remain in circulation because of their inability to aggregate in response to activated complement components; this allows such PMNs to avoid entrapment in the pulmonary vascular tree.

This subpopulation has been reported to have a bactericidal defect. Because the formation of oxygen reduction products is critical to the PMN-mediated microbicidal process, we chose to study the oxidative metabolism of these cells. The small number of cells available for study, however, limits the methodology employed. Luminol-enhanced chemiluminescence has been used to assess the formation of oxygen reduction products by small numbers of cells. Although the nature of the chemical reactions that allow PMN chemiluminescence are only poorly understood, it is clear that the formation of one or more oxygen reduction products is essential, since PMNs harvested from patients with chronic granulomatous disease of childhood do not emit light.

In this study, a significant decrease in chemiluminescence was observed among the intradialysis cells examined. This decrease occurred whether cells were responding to particle ingestion (i.e., opsonized zymosan) or soluble receptor-dependent stimuli (i.e., f-Met-Leu-Phe and PMA). A limited number of studies have investigated the oxidative metabolism of PMNs in association with chronic uremia and hemodialysis. Since the completion of our studies, Wissow et al. have reported a decrease in light emission among PMNs harvested 30 min after initiation of hemodialysis in response to S. aureus opsonized with either predialysis or dialyzed serum.

There are several possible explanations for the depressed chemiluminescence observed. First, it has previously been reported that myeloperoxidase is important in luminescence, and we have recently shown that this enzyme is critical to light detection in a luminol-enhanced system. Our failure to detect a difference in peroxidase concentration between pre- and intradialysis granulocytes makes this explanation unlikely. Furthermore, in two patients studied, H₂O₂ secretion by intradialysis cells was depressed, supporting the idea that these PMNs have a true defect in oxidative metabolism.

Next, we sought a biologic explanation for the abnormality observed. Preexisting stimulation or "exhaustion" of PMNs exposed to activated complement components might lead to defective cellular function. Baseline light emission values, however, were not elevated, nor were cells spontaneously polarized on initial examination. Nevertheless, it is certainly possible that these parameters returned to resting levels during the isolation and separation procedure.

We have previously shown that the binding of a
soluble stimulus can be correlated to the magnitude of the respiratory burst. More recently, Fletcher and coworkers have demonstrated a correlation between the binding of f-Met-Leu-Phe to PMNs and the secretion of $O_2$ and $H_2O_2$. In both these situations, an increase in the respiratory burst could be correlated to enhanced binding of a soluble stimulus. In order to study a situation in which the respiratory burst was depressed, we evaluated the binding of labeled formyl peptide to both pre- and intradialysis cells. Whereas normal control and predialysis PMNs had an equivalent number of receptors, fewer surface receptors were demonstrated on intradialysis cells. This difference in receptor number ($115 \times 10^3$/predialysis; $84 \times 10^3$ intradialysis) could be correlated with the 47% decrease in light emission observed with a saturating concentration of peptide. It is worth emphasizing that the polarization of intradialysis cells was normal. This observation is consistent with the report of Bass and colleagues in which stimulation of the respiratory burst required much higher concentrations of f-Met-Leu-Phe than did chemotaxis. It is also possible that greater receptor occupancy may be necessary for active chemotaxis than for the polarizing response.

The mechanism for the decrease in f-Met-Leu-Phe binding remains to be determined. It is possible that activated complement components have decreased the ability of the intradialysis PMNs to bind f-Met-Leu-Phe through a process of receptor “down regulation.” This process, however, is believed to be selective for the binding stimulus employed. Furthermore, Donnabedian and Gallin have recently reported that PMNs “deactivated” with C5a actually had an increased number of f-Met-Leu-Phe receptors relative to control cells. Down regulation of complement receptors in intradialysis cells could certainly explain their defective light emission in response to opsonized zymosan, where complement-mediated opsonization seems likely.

At this time we are unable to exclude more mundane explanations for the oxidative defect observed. It is possible that the cells we examined were senescent and soon to be removed from circulation. Alternatively, PMN contact with the dialysis coils per se might damage f-Met-Leu-Phe receptors and/or the oxidase which allows the reduction of oxygen and light emission. It should also be noted that this investigation was limited to uremic volunteers, and so the results cannot be directly applied to normal PMNs. Further studies will be necessary to establish the mechanism and general relevance of this defect.

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