Modulation of Multiple Neutrophil Functions by Preparative Methods or Trace Concentrations of Bacterial Lipopolysaccharide

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Human neutrophils were isolated from peripheral blood by four methods: 1) Ficoll–Hypaque gradients and erythrocyte lysis, 2) plasma–Percoll gradients, 3) a "lipopolysaccharide (LPS)-free" method yielding 85% neutrophils, and 4) by centrifugation of cells prepared by Method 3 through a plasma–Percoll gradient to produce pure neutrophils. The use of the Ficoll–Hypaque method resulted in spontaneous change of cell shape, enhanced formyl-methionyl-leucyl-phenylalanine (FMLP)-stimulated release of superoxide anion, increased release of lysosomal enzymes upon subsequent FMLP stimulation, and reduced chemotactic responsiveness, by comparison with the other methods. These effects were not due to erythrocyte lysis by NH_4Cl but were reproduced by exposure of neutrophils prepared by the "LPS-free" method or the use of plasma–Percoll gradients to 10–100 ng/ml LPS. Neutrophil change of shape and stimulated O_2^- production were particularly sensitive markers of these effects. The effects of trace concentrations of LPS in the modulation of neutrophil function may have relevance to the pathophysiology of endotoxemia and its resultant tissue injury. (Am J Pathol 1985, 119:101–110)

THERE have been reports that the isolation of neutrophils from peripheral blood may influence the subsequent function of the cells *in vivo* after radiolabeling and reinfusion.[1,2] Lane et al[1] showed that the subsequent circulation of rabbit neutrophils was greatly reduced as a result of *in vitro* manipulation during the isolation and labeling process, but there was no apparent effect on chemotaxis, enzyme secretion, or bacterial killing *in vitro*. However, there have been suggestions that neutrophil preparation procedures may influence the *in vitro* functions of the cells. Fearon and Collins[3] showed that neutrophil isolation induced a temperature-dependent increase in the expression of C3b receptors. In the development of a system to examine bacterial lipopolysaccharide (LPS) as a priming agent in neutrophil production of superoxide anion (O_2^-) following various stimuli,[4] LPS in 1–100 ng/ml concentrations was found as a contaminant of reagents used in standard methods of neutrophil preparation; and in order to avoid unacceptable endogenous priming, each step in the neutrophil preparation was required to be free of LPS. We therefore initiated a wider investigation of the effects of preparative methods on neutrophil function and into the effects of trace concentrations of LPS.

The study shows that preparative methods and trace concentrations of LPS may profoundly affect subsequent neutrophil function; and we identify markers which are particularly sensitive to these effects.

Material and Methods

Preparation of Human Neutrophils

Blood was drawn by venipuncture using a sterile °non-pyrogenic* gauge Butterfly needle (Abbott Hospital Products, North Chicago, Ill) and 50-ml °Plastipak dis-

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* The symbol ° denotes that the vessel or solution contained undetectable amounts of LPS (<0.1 ng/ml) by the amoebocyte lysate assay (Sigma Chemical Co., St. Louis, Mo).

posable polyethylene syringes (Becton Dickinson, Rutherford, NJ). In experiments in which the four preparation methods (see below) were compared, the Ficoll–Hypaque method consistently took an hour longer than the others, and in order to synchronize the completion of each method, we performed two venipunctures on the donor, in alternate arms, the first sample of less than 100 ml being taken 1 hour earlier. All steps, including centrifugation, were carried out at room temperature. Each preparation produced cell populations that were >98% viable by trypan blue exclusion, and cytocentrifuge smears were made of each for differential cell counts.

1. Standard Method, Using Ficoll-Hypaque Gradient and Erythrocyte Lysis

The cells were prepared as described by Newman et al.[5] By sterile procedures, venous blood was collected in 50-ml polyethylene tubes (Costar, Cambridge, Mass) containing 4.4 ml 3.8% or 5% citrate (Fisher Scientific, Pittsburgh, Pa) to a total volume of 40 ml. The tubes were centrifuged at 300*g* for 20 minutes. The platelet-rich plasma layer was aspirated and centrifuged at 2500*g* for 15 minutes to produce platelet-poor plasma (PPP), and 5 ml of 6% dextran (mol wt 500,000, Pharmacia, Piscataway, NJ) in sterile saline was added to the remaining contents of each tube. The final volume was adjusted to 50 ml with 0.9% NaCl (Fisher Scientific) in distilled water, mixed gently and thoroughly, and left for 30 minutes for erythrocyte sedimentation to occur. The leukocyte-rich plasma was aspirated and centrifuged at 275*g* for 6 minutes. The pellet was resuspended in 8 ml of PPP diluted 1:4 in saline and transferred to a 15-ml polystyrene tube (Corning, Corning, NY). With a sterile Pasteur pipette, the cells were underlayered with 3 ml Ficoll–Hypaque (density 1.077 g/ml, Pharmacia), and the gradients were centrifuged for 25 minutes at room temperature at 750*g*. The pellet, containing neutrophils and erythrocytes, was resuspended in an erythrocyte-lysing solution containing 0.155 M NH_4Cl. The neutrophils were then washed twice in Hanks' balanced salt solution with 0.25% bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, Mo, HBSA) without calcium and resuspended in HBSA with calcium. Cells prepared by this method were >95% neutrophils.

We have reported[4,6] that although the sterile plastic tubes did not contain adherent LPS in quantities detectable in the amoebocyte lysate assay, commercially prepared reagents in this assay usually contained contaminating LPS at concentrations between 1 ng/ml and 100 ng/ml. Several batches of freshly opened Ficoll–Hypaque (Ficoll–Paque, Pharmacia) and Ficoll were found to contain LPS.

2. Method Using Discontinuous Plasma–Percoll Gradients

This is a modification of the method described by Danpure et al,[7] but with slightly different densities and a two-step discontinuous gradient, so that erythrocytes pelleted at the base of the tube. A stock solution of Percoll (100% fine grade, Pharmacia) was prepared in °0.9% saline (Abbott Laboratories) in a ratio of 9:1 (vol/vol) Percoll/saline.

With sterile procedures venous blood was collected in °50-ml polyethylene tubes (Costar) containing 4.4 ml 3.8% or 5% °citrate (Fisher Scientific) to a total volume of 40 ml. The tubes were centrifuged at 300*g* for 20 minutes. The platelet-rich plasma layer was carefully aspirated and centrifuged at 2500*g* for 15 minutes for production of PPP. Five milliliters of 6% dextran (mol wt 500,000, Pharmacia) was added to the remaining contents of each tube, and the volume was made up of 50 ml with °0.9% saline, mixed gently and thoroughly and allowed to stand for 30 minutes for erythrocyte sedimentation to occur. The leukocyte-rich plasma was aspirated and centrifuged at 275*g* for 6 minutes, the pellet was resuspended in 2–3 ml PPP and transferred to a 15 ml °polystyrene tube, where it was underlayered with 2 ml freshly prepared 42% Percoll in PPP, which was in turn underlayered with 2 ml of freshly prepared 51% Percoll in PPP with the use of a siliconized Pasteur pipette, which had been sterilized by baking (240 C for 4 hours). The mixed leukocytes from up to 100 ml blood could be used for each gradient without overloading it. The gradients were centrifuged for 10 minutes at 275*g*. Mononuclear cells and some platelets remained at the top interface between plasma and the 42% Percoll layer, with neutrophils in a wider band at the interface of the 42% and 51% Percoll layers and extending into the 51% Percoll layer to a few millimeters above the erythrocyte pellet. Platelets could be removed from the mononuclear cells either by a further 5-minute 275*g* centrifugation step through 25% Percoll in PPP or by adding a third 25% Percoll in PPP step to the original gradient. Each band was aspirated with a °polyethylene transfer pipette (Fisher Scientific). It was found that an accurate preparation of the 51% Percoll step was needed to ensure complete separation of neutrophils and erythrocytes. The neutrophils from each gradient were then washed once in PPP and once in °Krebs–Ringer phosphate buffer Ph 7.23 with 0.2% dextrose (KRPD) prepared with dextrose in °0.9% sodium chloride solution. By this method the efficiency of neutrophil recovery from the gradient was >80%, and the neutrophil band was >95% pure, with 1–2% erythrocytes, 3–5% eosinophils and <0.5% mononuclear cells. The recovery of neutrophils by this method was quan-

titatively similar to the Ficoll-Hypaque method. There were no platelets visible in the neutrophil preparation.

3. "LPS-Free" Method

Cells were prepared by the method of Guthrie et al.[4] Vessels and solutions used did not have LPS detectable in the amoebocyte lysate assay (ie, <0.1 ng/ml LPS). Venous blood obtained using sterile procedure was anticoagulated with 10% °sodium citrate (3.8% solution in water, Fisher Scientific). Anticoagulated blood was added to 6% °dextran (mol wt 70,000) in 0.9% sodium chloride solution (Cutter Laboratories, Berkeley, Calif) in a 3:1 ratio (vol/vol, blood/dextran) and allowed to stand at room temperature for 1 hour. The leukocyte-rich plasma was aspirated and centrifuged, and erythrocytes were removed by hypotonic lysis with exposure to 10 ml 0.2% °sodium chloride in water for 15 seconds, with the immediate addition of 10 ml 1.6% saline with °dextrose 2 mg/ml (Fisher Scientific) prior to centrifugation. The cells were then washed twice in KRPD. Total cell recovery was similar to the plasma-Percoll method, although the purity of the neutrophils was lower, the final cell preparation containing 80–85% neutrophils, the remainder being lymphocytes and monocytes. In order to compare the effects of 0.155 M NH_4Cl lysing solution with hypotonic saline, in some preparations the erythrocyte lysis stage was performed with the use of 0.155 M NH_4Cl exposure for 15 seconds.

4. Method Producing Pure Neutrophils From Mixed Leukocytes Prepared by the LPS-Free Method

Because the "LPS-free" method (above) gave 80–85% neutrophils, a fourth preparation of cells was made by purification of these cells on a plasma-Percoll gradient (as in Method 2).

The leukocyte-rich plasma from the dextran sedimentation step of the preceding method was centrifuged for 6 minutes at 275*g*. The pellet was resuspended in 2 ml PPP, then underlayered with 42% Percoll, followed by 51% Percoll, and centrifuged for 10 minutes as in Method 2. The cell preparation contained >99% neutrophils, and erythrocyte lysis was not required. The cells were washed once in PPP and once in KRPD.

In Vitro Assays of Human Neutrophil Function

1. Change of Shape

At the completion of each preparation procedure 10^6 cells in 100 μl were mixed with 1 ml 2% cold glutaraldehyde. The fixed cells were examined microscopically at ×400 and were classified either as spherical or changed in shape. Five hundred cells were counted from each preparation. In order to assess the effects of LPS in the induction of neutrophil change of shape, we incubated 2.5×10^6 cells prepared by the LPS-free method at 37 C for 1 hour, either with 10 ng LPS in 1 ml KRPD or in KRPD alone. The cells were then fixed in cold 2% glutaraldehyde and classified as spherical or changed in shape.

2. Chemotaxis

Chemotaxis of neutrophils was performed in blind-end chambers (Celloplex, Basel, Switzerland) according to the method of Keller et al,[8] with the use of two wells separated by two membrane filters. Neutrophils (2.5×10^5 in 250 μl of 2% HBSA) were placed in the top well, and 230 μl of formyl-methionyl-leucyl-phenylalanine (FMLP) at 10 nM, 25 nM, or 100 nM or 2% HBSA was placed in the bottom well. These two fractions were separated by a 120-μ-thick membrane filter with an 8-μ pore size (cell side) and a 120-μ-thick filter with a 0.45 μ pore size (Schleicher & Schuell, Keene, NH) (chemoattractant side). The filled chambers were incubated for 20 minutes, 30 minutes, or 1 hour at 37 C in 5% CO_2. The reaction was stopped by fixing the filters in 20% butanol in ethanol, and the filters were stained with Weigart stain. All samples were run in triplicate. Chemotaxis was assessed either by the leading front method,[9] measuring the distance in microns that the leading front of cells (5 cells/high power field) had migrated into the 8-μ pore size filter, or by counting the numbers of neutrophils per high power field (mean of 5 fields for each filter) that had migrated 50 μ into the 8-μ pore size filter at 30 minutes. In experiments to assess the effect of added LPS on chemotaxis, neutrophils were prepared by the plasma-Percoll method, washed and resuspended in KRPD at 2×10^6 cells/ml. The cells were incubated for 1 hour at 37 C either with 10 ng/ml LPS or with buffer alone and resuspended in KRPD with 2% human serum albumin (shown to be LPS-free; a gift from Mr. John Hink, Cutter Biological, Emeryville, Calif) prior to the chemotaxis assay.

3. Superoxide Anion (O_2^-) Release With FMLP Stimulation

O_2^- was measured as superoxide dismutase-inhibitable reduction of cytochrome *c*, as described.[4] FMLP (Vega Biochemicals, Tucson, Ariz), 1 μM, was used as the stimulus; and the neutrophils were pretreated with either 10 ng LPS (extracted with phenol from *Escherichia coli* K235, a gift from Dr. Floyd MacIntyre, University of Colorado School of Dentistry) in KRPD or KRPD alone for 1 hour at 37 C.

4. Lysosomal Enzyme Release

Neutrophils from each preparation were suspended in a final volume of 1 ml KRPD containing 4×10^6 cells. Cytochalasin B (CB, Sigma Chemical Co.) was

dissolved in a mixture of dimethylsulfoxide (DMSO, Fisher Scientific) diluted with 9 vol of KRPD and added to the cells at a final concentration of 5 μg/ml for 5 minutes at 37 C. FMLP was dissolved in DMSO at 1 mM and diluted in KRPD to the appropriate concentrations. FMLP or KRPD was added to the reaction mixture and incubated at 37 C for 10 minutes. The cell suspensions were then centrifuged at 2500*g* for 10 minutes at 4 C, and supernatants were assayed for the release of myeloperoxidase (MPO), lysosyme, and lactic dehydrogenase (LDH), as previously described.[10] Release was expressed as a percentage of total cell-associated enzyme, which was determined by lysing control cells with 0.1% Triton X-100. In order to assess the effects of LPS, we preincubated the cells in 10 ng/ml LPS or in KRPD alone for 1 hour at 37 C prior to the addition of FMLP and assessment of enzyme release as above.

5. The Effects of NH_4Cl on Neutrophil Change of Shape and O_2^- Release

Neutrophils were prepared by the LPS-free method, but the erythrocyte lysis step was performed by exposing the cells to 0.155 M NH_4Cl lysing solution for 15 seconds. The cells were assessed at the completion of the preparation procedure and after 1-hour incubation in KRPD alone or KRPD and 10 ng/ml LPS for change of shape and FMLP-stimulated O_2^- release (as detailed above).

Results

Neutrophil Change of Shape

Neutrophils in suspension, when exposed to chemotactic factors, undergo a striking change in shape. Smith et al[11] showed that the change of shape induced by C5a or FMLP was associated with reduced motility and increased adhesiveness of cells to a glass surface. We therefore examined cells prepared by different methods to determine whether they exhibited spontaneous change in shape, which may have indicated "activation" of the cells as a result of the isolation procedure itself. We initially compared cells prepared by the Ficoll-Hypaque method with the plasma-Percoll method and, in all of eight observations, a higher percentage of cells prepared from the same donor by the Ficoll-Hypaque method exhibited "spontaneous" change of shape than cells prepared by the plasma-Percoll method (30.5% ± 15.8% versus 3.8% ± 3.6%; $P < 0.001$; Student *t* test). We then compared the four preparation methods. The appearance of the cells is illustrated in Figure 2 A–C, and Figure 3A demonstrates that in three experiments the only preparation of cells

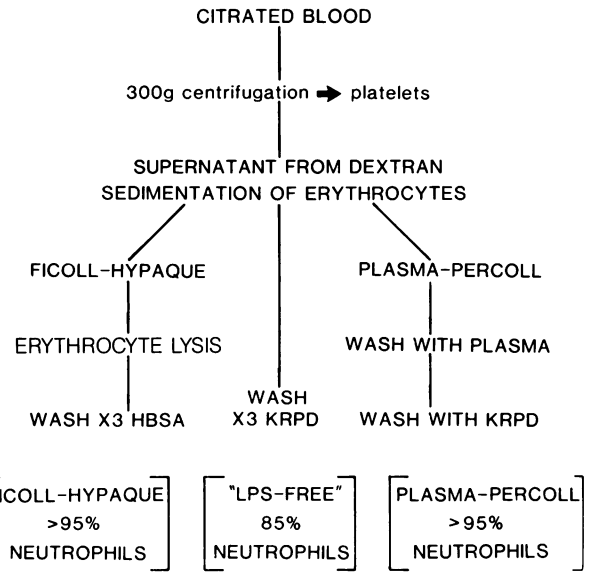

Figure 1—A simplified diagram of the preparative procedures. In addition (not shown), "LPS-free" neutrophils were centrifuged through a plasma-Percoll gradient to yield a population of cells which were > 95% neutrophils.

with a significant number of cells exhibiting change of shape was the Ficoll-Hypaque method. Because we had previously found LPS in concentrations of 1–100 ng/ml as a contaminant of reagents used in standard methods of neutrophil isolation, we next examined the effect of exposing neutrophils prepared by the "LPS-free" method to 10 ng/ml LPS for 1 hour at 37 C. It can be seen that incubation of neutrophils, prepared by the "LPS-free" method for 1 hour at 37 C, did not result in change of shape unless LPS was deliberately added (the same was true for cells prepared by the plasma-Percoll method). The addition of LPS resulted in up to 85% of cells exhibiting change of shape (Figure 3B; morphologic features are illustrated in Figure 2D). The same phenomenon occurred at 25 C, although fewer (about 30%) cells showed change of shape (data not illustrated).

Chemotaxis

Figure 4A shows the distance migrated by the leading front of cells in response to 10 nM FMLP, with the use of the four preparations of neutrophils in three consecutive experiments. Of the three donors used, Donor 1 was a poor chemotactic responder (60-minute assay shown), and Donor 2 and Donor 3 were moderate and good responders, respectively (20 minute assay shown for each). Cells prepared by the Ficoll-Hypaque method showed an 18–40% reduced chemotactic responsiveness, compared with those prepared by the other three methods.

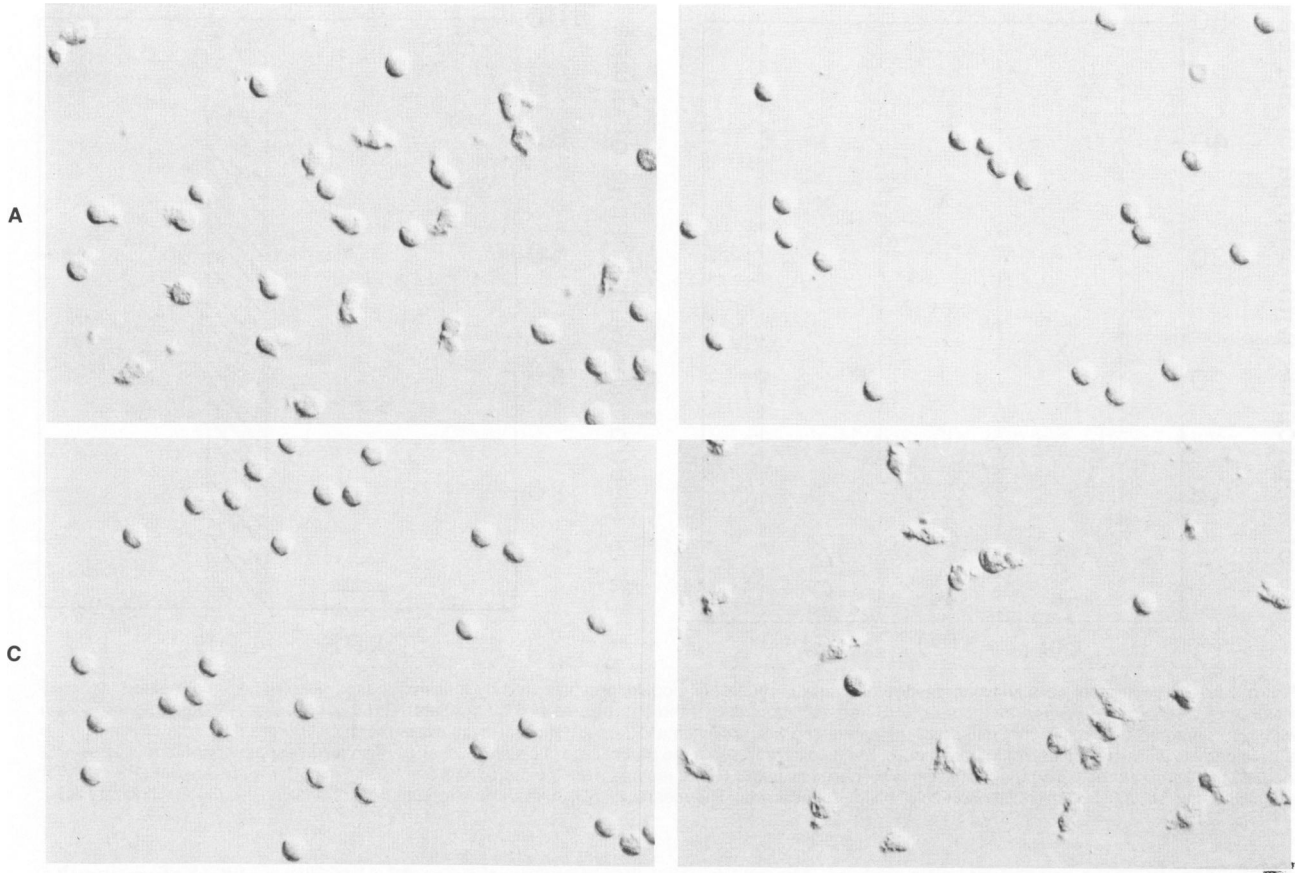

Figure 2—The appearance (× 400) of neutrophils prepared by the four different methods. **A**—The Ficoll–Hypaque method. **B**—The plasma–Percoll gradient method. **C**—The LPS-free method (80% neutrophils). **D**—Leukocytes prepared by the LPS-free method and exposed to 10 ng LPS/ml for 1 hour at 37 C. At the completion of each preparation 10^6 cells were immediately fixed in cold (4 C) 2% glutaraldehyde.

Figure 4B shows that preincubation of neutrophils with 10 ng LPS/ml for 1 hour at 37 C caused a significant reduction ($P < 0.005$) in chemotaxis (assessed by the number of cells which had migrated 50 μm into the 8-μ pore size filter at 30 minutes), as compared with cells incubated with buffer alone.

Superoxide Anion (O_2^-) Production

Figure 5 shows O_2^- release after 1 μM FMLP stimulation of neutrophils prepared by the four methods with three consecutive donors. Results are compared with and without pretreatment with 10 ng/ml of deliberately added LPS for 1 hour at 37 C. Neutrophils prepared by the Ficoll–Hypaque method appear primed for FMLP-stimulated O_2^- production and show little further priming on exposure to deliberately added LPS. This priming phenomenon has been shown by the addition of as little as 1–10 ng/ml exogenous LPS to leukocytes prepared by the LPS-free method.[4] The plasma–Percoll neutrophils show a further two- to threefold increase in release of O_2^- after deliberate exposure to LPS.

Enzyme Secretion

Neutrophils prepared by the Ficoll–Hypaque method gave two to three times more release of lysozyme after CB-enhanced stimulation with 10 nM FMLP, compared with other methods (Figure 6). Identical effects were seen with 25 nM and 50 nM FMLP for lysozyme release (n = 3; data not illustrated) and for myeloperoxidase (MPO) release in response to 10 nM, 25 nM, and 50 nM FMLP (n = 3, data not shown).

Exposure of cells prepared by the LPS-free method to 10 ng/ml LPS resulted in significant enhancement of lysozyme release upon subsequent stimulation with FMLP. In the absence of CB (not shown) neutrophils pretreated with LPS released 11.3% ± 0.9% (mean ± SEM) upon stimulation with FMLP, as compared with 6.5% ± 1.2% from cells pretreated with buffer alone (n = 5; $P < 0.01$, t test). Similar trends were noted for lysozyme release from cells preincubated with LPS, treated with CB, and stimulated with FMLP (15.3% ± 3% for preincubation with buffer versus 44.9% ± 2.9% for LPS pretreatment; n = 3). Similar results were obtained for MPO release. These data are comparable with

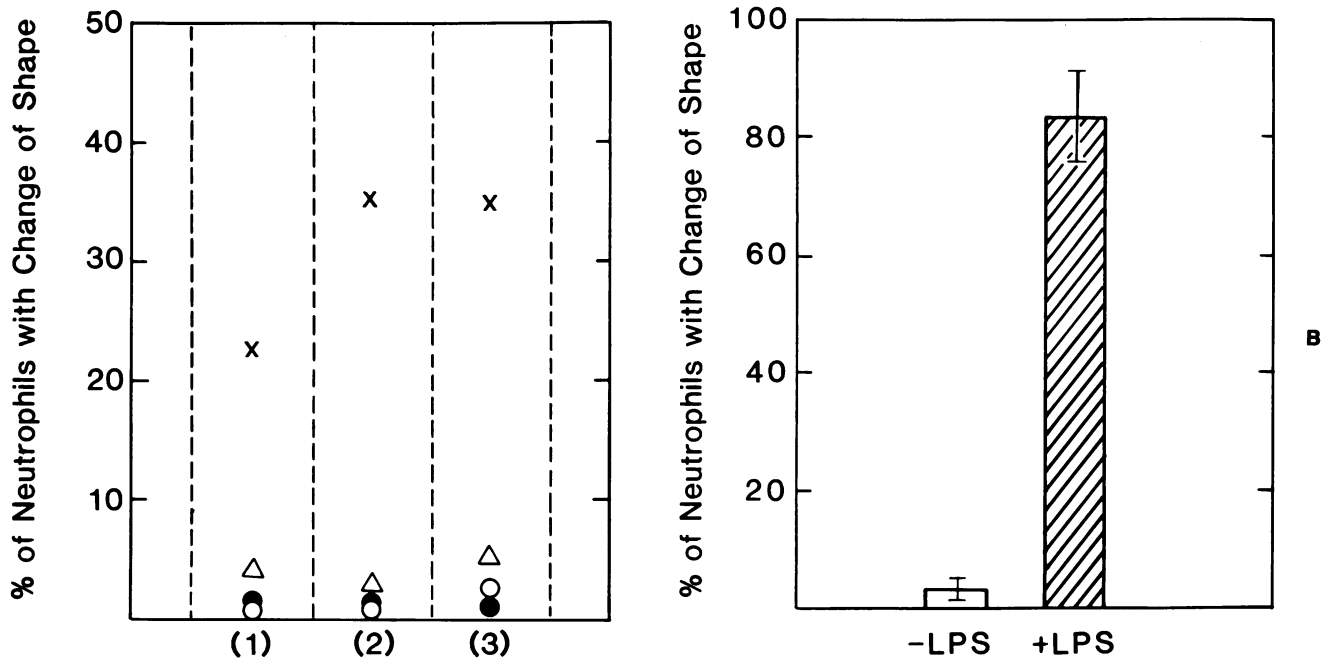

Figure 3A—Percentage of cells showing change of shape in neutrophil populations prepared by four methods: x, Ficoll–Hypaque method; ●, plasma–Percoll method; △, LPS-free method; ○, LPS-free cells centrifuged through plasma percoll gradient. Three consecutive experiments are shown with cells prepared by the four methods from the same blood sample with a different donor for each experiment. Cells were fixed immediately in 2% glutaraldehyde at the completion of the procedure. Each point represents the percentage of cells with change of shape in each preparation. **B**—LPS-induced neutrophil change of shape. Neutrophils prepared by the LPS-free method were incubated at 2.5 × 10^6/ml in buffer with 10 ng/ml LPS or buffer alone for 1 hour at 37 C before fixation in cold 2% glutaraldehyde. The means (± SD) from three experiments are shown ($P < 0.001$, Student t test).

those of Dahinden and Fehr,[13] who reported a coincubation effect of LPS in FMLP-stimulated lysosomal enzyme release. During the above assays spontaneous LDH release was 4–8%, without significantly higher release from cells prepared by the Ficoll–Hypaque method or from cells pretreated with LPS.

NH_4Cl Effects on Neutrophil O_2^- Release and Change of Shape

It has been reported that weak bases, including NH_4Cl, can activate macrophages, as assessed by lysosomal enzyme release[14]; but Klempner and Styrt have recently shown that NH_4Cl inhibits degranulation of human neutrophils.[15] We considered it important to assess whether functional differences in neutrophils prepared by the Ficoll–Hypaque method might, in part, have been a result of the erythrocyte lysis step which employed 0.155 M NH_4Cl. Figure 7A shows that the use of NH_4Cl in the erythrocyte lysis step resulted in neutrophil change of shape at the end of the preparation process, but that after 1-hour incubation at 37 C the cells had recovered completely to control levels. Thus, the initial change of shape could not have been a result of an LPS-like effect of the NH_4Cl (see Figure 7B). By contrast, cells prepared by the Ficoll–Hypaque method showed only partial recovery in shape after 1-hour incubation at 37 C (66% ± 8% before incubation to 31% ± 4% after incubation). This result is consistent with the premise that part of the neutrophil change of shape at the completion of the Ficoll–Hypaque preparation may have been attributed to NH_4Cl, but part remained after 1-hour incubation, which was consistent with an LPS effect.

Figure 7B shows that exposure of neutrophils to NH_4Cl does not itself result in greater release of O_2^- after FMLP (ie, it does not have an LPS-like effect) and does not significantly affect the priming induced by the deliberate addition of LPS.

Discussion

In the comparison of preparative methods, there were no significant differences observed in neutrophil function *in vitro* between neutrophils prepared with the use of plasma–Percoll gradients, leukocytes (80–85% neutrophils) prepared by an "LPS-free" method, and LPS-free leukocytes centrifuged through a plasma–Percoll gradient to yield pure neutrophils. However, the neutrophils prepared by the Ficoll–Hypaque method showed spontaneous change of shape, reduced chemotactic responsiveness, and increased production of O_2^- and lysosomal enzymes upon subsequent stimulation with FMLP, by comparison with the other methods.

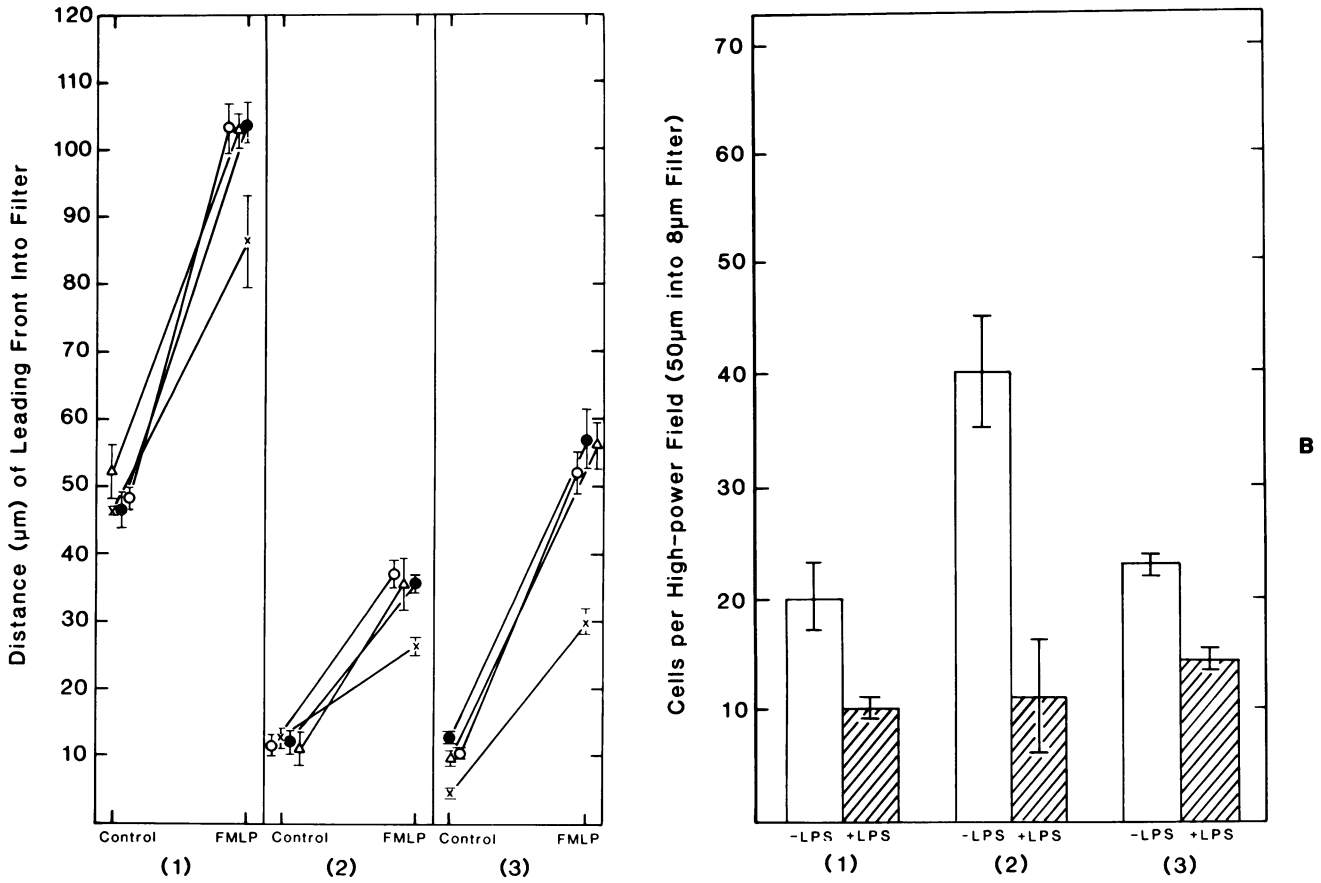

Figure 4A—Chemotaxis to 10 nM FMLP of cells prepared by different methods (see Figure 3 for symbols). Three consecutive experiments are shown with cells prepared by the four methods from the same blood sample using a separate donor for each experiment. Each point represents the mean ± 1 SD of three experiments, showing the distance in microns that the leading front (4–5 neutrophils/× 400 high power field) migrated into the 120-μ deep, 8-μ pore size filter in response to buffer or 10 nM FMLP for 60 minutes (Experiment 1) or 20 minutes (Experiments 2 and 3) at 37 C in 5% CO_2. In each experiment the neutrophils prepared by the Ficoll–Hypaque method migrated significantly less well in response to FMLP than did cells prepared by the other methods ($P < 0.01$ for each experiment, Student t test). **B**—The effect of LPS on neutrophil chemotaxis. Neutrophils prepared by the plasma–Percoll method were incubated at 2 × 10^6/ml with 10 ng/ml LPS (*hatched bars*) or in buffer alone for 1 hour at 37 C prior to the chemotaxis assay. Chemotaxis to 10 nM FMLP was assessed by the number of neutrophils per high power field (mean of five fields) that had migrated 50 μ into the 8-μ pore size filter at 30 minutes. Three experiments are shown, and each point represents the mean (± SD) of results with three filters. LPS pretreatment caused a significant reduction in chemotaxis ($P < 0.005$, t test, for means of all three experiments).

Cells prepared by the Ficoll–Hypaque method were viable, as assessed by exclusion of trypan blue and release of LDH, and we believe this study indicates that the cells are in a "primed" or activated state, rather than being functionally impaired.

Guthrie et al[4] have shown that the exposure of neutrophils to LPS in concentrations as low as 1–10 ng/ml "primed" the cells to release more O_2^- upon subsequent stimulation with FMLP. However, in order to detect a priming effect at such low concentrations, reagents in their isolation system needed to be free of LPS. It was found that reagents, including Ficoll–Hypaque, used in standard methods of neutrophil isolation contained 1–100 ng/ml LPS.[4,6] In the present study, by using isolation methods that reduced LPS contamination, we were able to show that 10 ng/ml LPS added deliberately to the cells resulted in neutrophil change of shape and reduced chemotactic responsiveness, thus reproducing the effects of neutrophil isolation by the Ficoll–Hypaque method.

As we have previously reported that reagents used in standard neutrophil isolation methods may contain LPS (1–100 ng/ml)[4,6] and have shown that neutrophils, isolated by methods which reduce LPS contamination, are exquisitely sensitive (particularly in the change of shape assay and the priming assay for FMLP-stimulated O_2^- release) to the effects of deliberately added LPS in trace concentrations, it is a reasonable premise that contaminating LPS was the major cause of differences observed between isolation methods. However, it is possible that other factors may have contributed to the differences observed:

1) Ficoll–Hypaque may have other effects on cells, including osmotic effects,[16] and it is possible that the

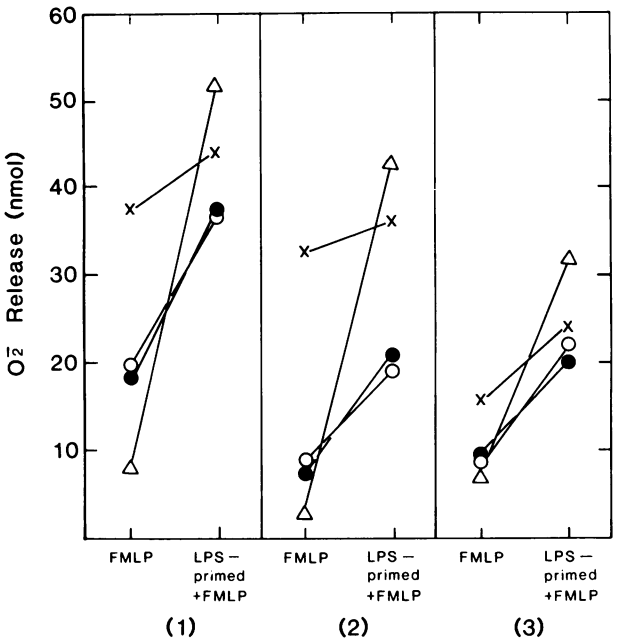

Figure 5—O_2^- release with and without LPS priming of neutrophils prepared by the four methods (see Figure 3 for symbols). Three consecutive experiments are shown with cells prepared by the four methods from the same blood sample, with a separate donor for each experiment. Each point represents the mean release of triplicate samples preincubated in 10 ng/ml LPS or in buffer alone for 60 minutes followed by stimulation with 1 μM FMLP for 10 minutes. There was a significant difference in O_2^- release (Student *t* test) without deliberate LPS priming, in a comparison of neutrophils prepared by the Ficoll–Hypaque method with the LPS-free method ($P < 0.001$) or the plasma–Percoll method ($P < 0.005$), or LPS-free cells centrifuged through plasma–Percoll ($P < 0.001$).

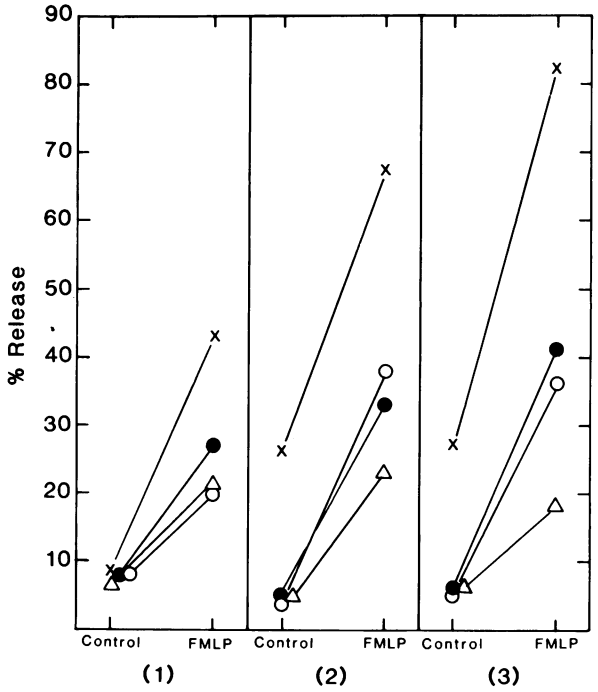

Figure 6—FMLP-stimulated lysozyme release from neutrophils prepared by the four methods (see Figure 3 for symbols). Three experiments are shown with cells prepared by the four methods from the same blood sample, with a separate donor for each experiment. Each point represents the mean lysozyme release of samples assayed in duplicate with 4×10^6 cells incubated with 100 nM FMLP (Experiment 1), 50 nM FMLP (Experiments 2 and 3), or buffer control for 10 minutes in the presence of cytochalasin B, 5 μg/ml. There was a significant difference (Student *t* test) in lysozyme release after FMLP stimulation when neutrophils prepared by the Ficoll–Hypaque method were compared with neutrophils prepared by the LPS-free method ($P < 0.001$) or the plasma–Percoll method ($P < 0.005$) or LPS-free cells centrifuged through plasma–Percoll ($P < 0.01$).

long-chain carbohydrate moiety of Ficoll may have LPS-like effects on cells.

2) Erythrocyte lysis was necessary in the Ficoll–Hypaque preparation. Erythrocyte-lysing solutions often contain the weak base NH_4Cl, which has been shown to stimulate macrophages *in vitro*.[13] However, exposure of neutrophils prepared by the "LPS-free" method to NH_4Cl did not produce the effects of the Ficoll–Hypaque preparation method, as assessed by neutrophil change of shape and FMLP-stimulated O_2^- release. In addition, it has been reported that the effect of NH_4Cl on neutrophils is to inhibit rather than enhance degranulation.[15]

3) In the plasma–Percoll method the neutrophils remained in contact with autologous plasma throughout the isolation process, and it is possible that this protected the cells in some fashion. Indeed, due to interference in the limulus lysate assay, it was not possible to be certain that Percoll was LPS-free, and it is feasible that high density lipoproteins in the plasma may have bound trace amounts of LPS.[17] In addition, Percoll, by contrast with Ficoll–Hypaque, has a low osmolality, enabling gradients with physiologic osmolalities to be made, and its low viscosity results in rapid cell separation and a shorter gradient centrifugation time. Although the increased length of the Ficoll–Hypaque procedure may have contributed to an LPS-like priming effect, this cannot be the explanation for the differences observed between the cell preparations because neutrophils prepared by the "LPS-free" or plasma-Percoll methods did not undergo spontaneous change of shape or priming for FMLP-stimulated O_2^- release when incubated for 1 hour in buffer alone.

The importance of this study is that it shows that neutrophil function may be influenced by cell isolation procedures, and, with the minute concentrations of LPS that have now been demonstrated to affect neutrophil and monocyte[6] function, it raises concern that LPS may be a confounding factor in some *in vitro* systems, making comparisons with processes *in vivo* difficult until the modulating roles of trace concentrations of LPS are fully elucidated.

The observations may also have relevance for the *in vivo* functions of isolated neutrophils upon subsequent reinfusion. We have observed that rabbit neutrophils, prepared by the Ficoll–Hypaque method, failed to cir-

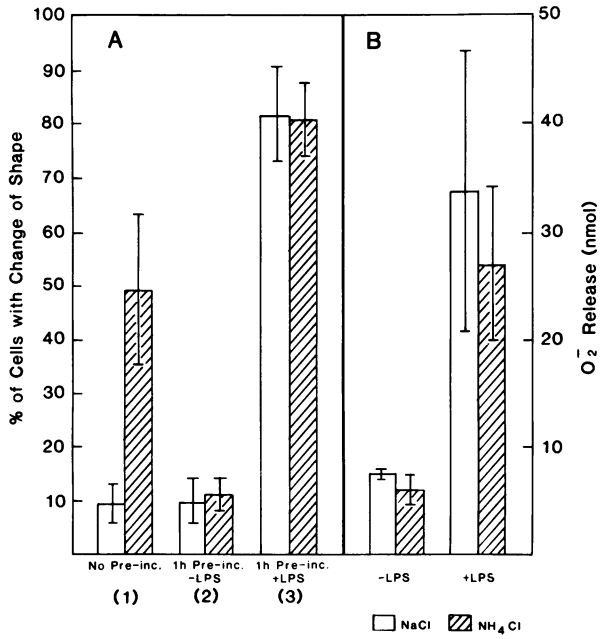

Figure 7—The effect of NH_4Cl erythrocyte lysis on neutrophil change of shape and FMLP-stimulated O_2^- release. Cells were prepared by the LPS-free method. The means (± SD) of three experiments are shown. **A**—Percentage of neutrophils with change of shape after lysis with 0.155 M NH_4Cl (*hatched bars*), compared with hypotonic saline: 1) percentage of neutrophils with change of shape at the completion of the preparation process; 2) percentage of neutrophils with change of shape after 1-hour incubation in buffer alone; and 3) percentage of neutrophils with change of shape after incubation in buffer with 10 ng/ml LPS. **B**—Neutrophil O_2^- release after erythrocyte lysis with 0.155 m NH_4Cl (*hatched bars*), compared with hypotonic saline. In each experiment triplicate samples were preincubated with 10 ng/ml LPS or with buffer alone for 1 hour, followed by stimulation with 1 μM FMLP for 10 minutes.

culate effectively after reinfusion, whereas rabbit neutrophils, prepared using plasma-Percoll gradients, circulated effectively, but their circulation could be markedly reduced by *ex vivo* exposure to trace concentrations of LPS (unpublished data). Because radiolabeled neutrophils, isolated from peripheral blood, are now being used in the investigation of human disease,[18] it is important that the cells are not "activated" during the isolation and labeling procedure. In addition, granulocyte transfusions have been used in patients with granulocytopenia induced by cytotoxic agents.[19]

The results of this study show that LPS in very low concentrations has direct effects (in the absence of plasma) on neutrophil function *in vitro*, but the pathophysiologic relevance of these effects remains to be proven. Neutrophil change of shape occurs in response to chemotactic peptides.[11,12] In this situation, it occurs rapidly and is closely associated with increased adhesiveness of the cells,[11] but the effect is evanescent.[12] However, the neutrophil change of shape we demonstrated after exposure to 10 ng/ml LPS was prolonged, with a slower onset, and was more temperature-dependent, requiring 1-hour exposure at 37 C for maximal response, although a smaller effect was seen by 30 minutes or at 25 C (unpublished data). The reduced chemotactic responsiveness we observed in neutrophils treated with 10 ng/ml LPS may have been a result of increased adhesive properties of the cells.[13] The study also shows that neutrophils, preexposed to trace concentrations of LPS, release greater quantities of lysosomal enzymes upon subsequent stimulation with chemotactic peptides, and we have previously reported an identical effect for O_2^- release.[4] These results are similar to those reported by Dahinden and Fehr,[13] who coincubated the cells with LPS. Previous reports of the effects of LPS, often in very high concentrations, on neutrophil function have been variable and sometimes contradictory (as we have previously discussed in more detail[4]).

It is likely that these *in vitro* observations are relevant to the pathophysiology of endotoxemia. In its early stages, endotoxemia causes prolonged neutropenia and sequestration of cells in vascular beds[20]; and the effects of LPS, in the induction of neutrophil change of shape, increased adhesiveness, and reduced chemotactic responsiveness may be causally associated with these phenomena. Endotoxemia can result, at a later stage, in tissue injury, such as pulmonary vascular injury,[21] which may be neutrophil-dependent.[22] It is possible that the neutrophil "priming" effect of LPS in concentrations of 1–100 ng/ml (comparable with circulating levels in human endotoxemic states[23]), which modulates neutrophils to release greater quantities of O_2^- and lysosomal enzymes upon subsequent stimulation with chemotactic peptides which have been demonstrated to be generated in endotoxemia,[24] would markedly enhance local tissue injury.

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