Membrane-bound Lactoferrin Alters the Surface Properties of Polymorphonuclear Leukocytes

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ABSTRACT Polymorphonuclear leukocytes (PMN) aggregate and avidly attach to endothelium in response to chemotactic agents. This response may be related in part to the release of the specific granule constituent lactoferrin (LF). We found by using immunohistology and biochemical and biophysical techniques that LF binds to the membrane and alters the surface properties of the PMN. Upon exposure of PMN treated with 5 μg/ml cytochalasin B to 2×10^{-7} M formyl-methionine-leucine-phenylalanine for 5 min, the PMN mobilized LF to their surface as observed by immunoperoxidase staining for LF. At added LF levels ranging from 4 to 15 μg/10^7 PMN there was a dose-dependent reduction in PMN surface charge reaching 4 mV, when the partitioning into the membrane of a charged amphipathic nitroxide spin label was measured by electron spin resonance spectroscopy, whereas transferrin was without effect. When ^{125}I-FeLF was added to human PMN in increasing amounts and the results corrected for the residual amount of free LF contaminating the cells, the PMN were saturated with LF at concentrations between 100 and 200 nM in the medium. Human PMN bound 1.35×10^6 molecules per cell and the calculated value for the association constant for these receptors was 5.2×10^6 M^{-1}. Additionally, 6 μg/ml LF served as an opsonin for rabbit PMN to promote PMN uptake by rabbit macrophages, when assessed by electron microscopy, but lysozyme did not. These studies indicate that LF can bind to the surface of the PMN and reduce its surface charge. This correlates with enhanced "stickiness" leading to a variety of cell-cell interactions.

INTRODUCTION

After intravenous infusion of chemotactic factors into animals, polymorphonuclear leukocytes (PMN)[1] have been observed to marginate along vessel walls (1–6). Pulmonary sequestration of PMN along with neutropenia often occurs. The neutropenia is a transient phenomenon that parallels the ability of the chemotactic factors to induce PMN aggregation in vitro (7, 8). Because PMN more readily release specific granule contents compared with primary granule constituents upon exposure to chemotactic factors, a causal relationship has been suggested to exist between specific granule discharge and PMN "stickiness" (9–11). Recently we found that lactoferrin, a product of specific granules, could promote PMN aggregation and enhance their attachment to endothelial cells in vitro (11). Additionally, lactoferrin was found to promote the attachment of PMN to endothelial vessel linings in vivo when monitored visually and to induce neutropenia (12). These observations suggest that a direct relationship exists between the externalization of lactoferrin to the plasma membrane and PMN "stickiness." This study documents that exogenous lactoferrin avidly binds to the PMN surface and reduces its surface charge, which in turn may promote PMN attachment to other cellular surfaces.

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[1] *Abbreviations used in this paper*: BSA, bovine serum albumin; CAT_{12}, 4-(dodecyl-dimethyl ammonium)-1-oxyl-2,2,6,6-tetramethyl-piperidine bromide; ESR, electron spin resonance spectroscopy; K_a, association constant; K_d, dissociation constant; KRPG, Krebs-Ringer phosphate, pH 7.4, with 5 mM glucose; LF, lactoferrin; PBSG, phosphate-buffered saline, pH 7.2, with 5 mM glucose; PMN, polymorphonuclear leukocytes.

METHODS

Isolation of human PMN. Human PMN (98% PMN with less than one platelet per 1,000 cells) were isolated from heparanized venous blood drawn from consenting normal human volunteers following approval of the Indiana University Committee for Protection of Human Subjects. After dextran sedimentation, the leukocyte-enriched plasma was layered on Ficoll-Hypaque and centrifuged at 400 *g* for 30 min at 4°C (13). The supernatant fluid was discarded and the cell pellet exposed to distilled water for 20 s to free the PMN of contaminating erythrocytes, after which isotonicity was restored. PMN were resuspended at a concentration of 1×10^7/ml in Krebs-Ringer phosphate, pH 7.4, containing 5 mM glucose (KRPG).

Immunoperoxidase staining. Smears of peripheral blood PMN were fixed in either buffered formol acetone (20 mg Na_2HPO_4, 100 mg KH_2PO_4, 45 ml acetone, 25 ml concentrated formalin, 30 ml distilled water) for 30 s at room temperature or in buffered solution (lacking acetone), and rinsed in distilled water. After fixation the PMN were exposed to methanol containing 0.3% hydrogen peroxide for 30 min to block endogenous peroxidase activity followed by washing three times in Tris-buffered saline, pH 7.6. Rabbit anti-human lactoferrin (Dako Accurate Chemical Co., Hicksville, NY) at a 1:100 dilution was applied for 30 min and then removed by washing three times in Tris-buffered saline. In the experiments using buffer lacking acetone, the PMN were initially treated with 5 μg/ml cytochalasin B and then stimulated with 2×10^{-7} M FMLP at 37°C for 5 min, pelleted, and then processed in the buffer lacking acetone as above. After the addition of the lactoferrin antibody, swine anti-rabbit immunoglobulin (Dako Accurate Chemical Co.) at a 1:50 dilution was added for 30 min and removed by washing three times. A rabbit anti-horseradish peroxidase:horseradish peroxidase complex was added for 30 min and the complex was developed by washing in Tris-buffered saline containing 0.5 mg/ml diaminobenzidine (Polysciences, Inc., Warrington, PA) and 0.01% hydrogen peroxide for 60 s (14). The slide was washed in tap water for 5 min and then counter-stained with Gills hematoxylin and washed again in tap water, dehydrated, and mounted with a permount solution. Controls consisted of adding nonimmune serum instead of specific antibody.

Spin labeling. To quantitate the relative surface charge of variously treated cell samples, the partitioning of a positively charged, amphipathic, nitroxide spin label between the hydrophobic environment of the PMN membranes and the aqueous buffer was measured (15, 16). Experiments used multiple samples and controls so that statistical methods could be used to isolate the variable being investigated. To minimize the effects of isolation procedures on cell surface charge, electron spin resonance spectroscopy (ESR) experiments used PMN isolated solely by two hypotonic lysis cycles. Greater than 75% of the cells were PMN, with mononuclear cells comprising the remainder. Final cell supensions were in phosphate-buffered saline, pH 7.2, containing 5 mM glucose (PBSG) at a concentration of 5.5×10^7 PMN/ml. Plastic materials were used throughout and the experiments were completed within 4 h to minimize aging effects on PMN surface charge. For a typical experiment, 100 μl of suspended cells were aliquoted into individual 12 × 75-mm polystyrene tubes and kept on ice. In a timed sequence, each sample was brought to a final concentration of 1.5 mM Ca^{++} and 1 mM Mg^{++}, vortexed and placed in a 25°C water bath. After a 10-min incubation period, appropriate additions of either PBSG, control solution (dimethylsulfoxide in PBSG) or stimulant (formyl-methionine-leucine-phenylalanine, FMLP, rabbit lactoferrin, or human transferrin) were made followed by vortexing. After a further 4-min incubation, the spin label 4-(dodecyl-dimethyl ammonium)-1-oxyl-2,2,6,6-tetramethyl-piperidine bromide (CAT_{12}; Molecular Probes, Plano, TX) was added to a final concentration of 30 μM. After a 2-min incubation period, which was sufficient for the partitioning of the CAT_{12} to reach equilibrium, each sample was transferred to a glass capillary and centrifuged (45 S, 1,500 *g*, 25°C). The pellet of cells was broken off and the capillary resealed with hematocrit tube sealant. The capillary was then used for measurement of the CAT_{12} remaining free in solution. In some experiments the capillary tubes were not centrifuged to remove the cells. This allowed measurements of CAT_{12} bound to the cell membrane and of the rate of reduction of the nitroxide moiety by the cells. All samples, including cell-free samples for quantitation of total spin label added, were treated identically throughout.

ESR data acquisition and analysis. The partitioning of charged nitroxide spin probes has been used to measure the surface potential of artificial vesicles (16, 17), purple membranes (18), and mitochondria inner membranes (15, 19) and was adapted herein for intact PMN. ESR spectra of CAT_{12} bound to the PMN membrane and free in the aqueous phase were obtained on a standard balanced-bridge ESR spectrometer with diode detection operating at 9.1 GHz. Operating conditions and instrument settings were as described previously (20), except the peak-to-peak modulation amplitude was 1.0 G and the field sweep was 50 G. Two spectra from each sample were recorded and the results averaged. All samples were coded and read blind. All statistical procedures were done according to accepted algorithms using published statistical tables (21). All statistical results are expressed as *P* values, where *P* is the probability that the null hypothesis is true.

The observed partitioning (P) of a probe molecule is due to both thermodynamic and electrostatic forces and is measured experimentally as the ratio of probe molecules free in solution to those bound to the cell membrane. The relative change in the partitioning due to addition of exogenous factors can be related to changes in the cell surface potential by the Boltzmann relation (15, 16).

$$\Delta\psi_s = \frac{kT}{ze} \ln \frac{P_1}{P_2}, \tag{1}$$

where P_1 and P_2 are the partitioning of CAT_{12} in stimulated and resting cells respectively, z is +1 for the positively charged CAT_{12}, e is the electronic charge, k is the Boltzmann constant, and T is the absolute temperature. An assumption inherent in the use of Eq. 1, that the observed change does not involve altered thermodynamic properties of the membrane, was accepted when no significant alteration in lipid fluidity was detected. As measured by the spin label 5-doxyl stearic acid (20), the membrane order parameters of intact PMN in suspension treated with 60 μg/ml lactoferrin, 60 μg/ml transferrin, or buffer were 0.653, 0.655, and 0.654, respectively. The surface potential, ψ_s, can be related to the surface charge density and the ionic composition of the buffer by the Guoy-Chapman theory (22). In control experiments where the ionic composition of the buffer was varied, the data agreed with theoretical calculations when a surface charge density of one charge per 4 nm^2 was used, even though at some ionic strengths aggregation was observed. This agreement demonstrates that the method can detect changes in the cell surface potential, even in the presence of agents that induced aggregation.

Preparation of labeled lactoferrin (LF). Human milk LF

was purchased from Sigma Chemical Co. (St. Louis, MO) and further purified to homogeneity by copper chelation chromatography as previously described (11). Under the conditions used for the purification of LF in medium devoid of calcium and in subsequent binding assays (23), the LF did not appear to form aggregates when applied to a cationic gel run under nonreducing conditions (11). In brief, its purity was assessed by polyacrylamide gel electrophoresis using a 7 × 75-mm column. A single band was observed at 78,000 mol wt. Additionally immunoelectrophoresis revealed that the LF reacted with anti-LF, but not with antibodies directed against granule lysozyme or cationic protein (11). Iron-saturated LF was prepared by adding ferric ammonium citrate in the presence of bicarbonate (24), and dialyzing against double-distilled water for 24 h at 4°C.

Bound iron was calculated from the absorbance at 450 μm, using the value $E^{1cm}_{450} = 0.547$ for the extinction coefficient, and lactoferrin was found to be 80% iron-saturated. Approximately 5–10-mg samples of FeLF were labeled with 1 mCi iodine-125 (100 mCi/ml, Amersham Corp., Chicago, IL) by the chloramine-T method (25). Free iodine was separated from the protein by gel filtration on Sephadex G-50 (Pharmacia Fine Chemicals, Uppsala, Sweden). Iodine-labeled proteins were stored at −20°C in aliquots containing 0.5% albumin before use.

Binding assay. Binding of lactoferrin to intact cells was measured by using 1.0×10^7 cells/ml in a total volume of 0.1 ml of PBS at pH 7.5, containing 2 mg of bovine serum albumin (BSA)/ml. Except where noted, the incubation of cells with ^{125}I-labeled Fe-saturated human LF was carried out with gentle shaking for 45 min at 4°C to prevent possible PMN aggregation and internalization of LF. Binding was stopped by rapid dilution and chilling on ice with 2 ml of cold PBS/BSA. The cell mixture was immediately centrifuged at 800 *g* for 10 min at 4°C. The cell pellets were washed two times with cold PBS/BSA and were counted for radioactivity in a gamma counter (Beckman Instruments, Inc., Palo Alto, CA). The percentage of ^{125}I-FeLF bound to the cells was calculated. Background binding was determined by running a control without cells. The specific binding was determined by subtraction of nonspecific binding obtained from controls containing cells and 10-fold excess unlabeled iron-saturated LF. Specificity of binding was further determined by adding human transferrin or IgG at 10-fold excess to the tubes containing labeled FeLF.

In separate experiments 12 μg/ml cold LF was added to 1×10^7 PMN/ml at either 37° or 4°C for 40 min to determine whether exogenous LF could provoke PMN degranulation (11). Under these conditions there was no measurable release of the specific granule constituent, lysozyme, which indicates that degranulation was not occurring.

Scatchard analysis. To demonstrate the existence of receptors and the average receptor's affinity for LF on human PMN, the number of receptors was calculated by determining the binding of various amounts of ^{125}I-iron-saturated LF to a fixed number of cells under equilibrium conditions (26, 27). The molecules of LF bound to one cell (r) and the molecules of LF free in the surrounding medium (c) were calculated for each experimental point by using Avogadro's number and a molecular weight of 78,000 for LF. A Scatchard plot of r/c vs. r was constructed, with the relation $r/c = nK - rK$. The value n, or the x-intercept, represented the amount of LF molecules on each cell at saturation. The slope of the line, K, represented the effective association constant and was expressed in liter/mole. All curve fitting (by the least squares method) and subsequent analysis of data was done by computer.

Preparation of rabbit PMN and alveolar macrophages for ultrastructural studies. PMN were obtained from rabbits by peritoneal lavage 18 h after injection of 12% sodium caseinate (28). The macrophages were obtained from the same rabbit by a normal saline lung lavage at the time the peritoneal PMN were harvested (29). Both PMN and alveolar macrophages were suspended in KRPG and pelleted by centrifugation at 250 *g* for 5 min at room temperature. The cells were washed twice in KRPG and adjusted to a final concentration of 1×10^7 cells/ml.

To obtain a time and dose response of alveolar macrophages phagocytizing LF and lysozyme-coated PMN, PMN were incubated with 25 μg of one of the above proteins for 15 min at 37°C and then pelleted at 200 *g* for 10 min at 4°C. The treated PMN were resuspended in KRPG and alveolar macrophages were then added at a ratio of 1:3 i.e., macrophage:PMN, and incubated at 37°C. Aliquots of cells were then removed at time points of 0, 5, 15, and 30 min and fixed in 3% gluteraldehyde in 0.1 M sodium cacodylate, pH 7.4, for 1 h at room temperature. Optimal ingestion was determined to occur at 15 min. The dose response was obtained by incubating aliquots of PMN with 3, 6, and 12 μg of either LF or lysozyme for 15 min at 37°C. Alveolar macrophages were then added to PMN at a ratio of 1:3. After a 15-min incubation at 37°C all cells were fixed in a manner identical to that stated above.

After three 5-min washes with 0.1 M sodium cacodylate buffer, pH 7.4, all cells were postfixed in 1% OsO_4 in 0.1 M sodium cacodylate, pH 7.4, for 1 h at room temperature. After another series of three buffered washes the cells were stained en bloc in 0.5% aqueous uranyl acetate at 4°C overnight. The cells were dehydrated in ethanol and embedded in Spurr resin (30). All samples were studied by an unbiased observer in a double blind fashion. 100 alveolar macrophages were viewed under low magnification using the electron microscope. Each alveolar macrophage was examined for the presence of phagocytized PMN. The percentage of ingesting macrophages and the number of PMN internalized per 10 macrophages were determined (31). To determine if exogenous LF or lysozyme could modify the macrophage phagocytic rates per se, alveolar macrophage phagocytosis of C3-coated lipopolysaccharide-paraffin-oil droplets in the presence of 12 μg/ml added protein was determined over 10 min (31). Statistical analysis was determined by analysis of variance.

RESULTS

PMN aggregate and avidly attach to endothelium in response to chemotactic agents. This response can be related in part to the release of the specific granule constituent LF (11). To assess whether LF is translocated to the surface of the PMN during activation in response to the chemotactic peptide, FMLP, we used the immunoperoxidase technique to localize the specific granule product LF. In resting cells exposed to buffer containing acetone, which allows penetration of the antibody, LF was distributed throughout the cytoplasm of the cell (Fig. 1a) compared to the lack of activity in cells not treated with acetone (Fig. 1b). In contrast, in cytochalasin B-treated cells activated with FMLP, LF was localized to the perimeter of the cell in medium devoid of acetone (Fig. 1c) and LF

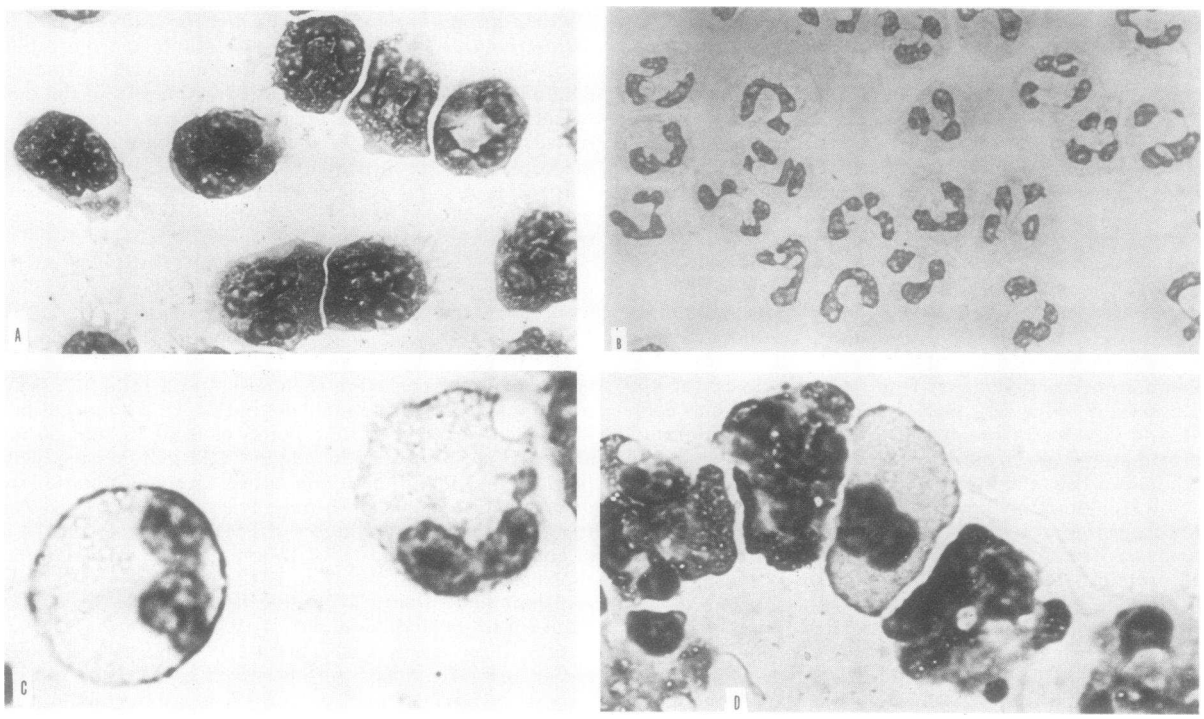

FIGURE 1 Immunoperoxidase stains of PMN. (A) Normal adherent PMN treated after fixation in acetone and inhibition of myeloperoxidase, with horseradish-peroxidase-labeled antilactoferrin followed by diaminobenzidine/H_2O_2 substrate. (B) PMN were treated as above but in medium devoid of acetone. (C) PMN were treated with 5 μg/ml cytochalasin B and then stimulated with 2×10^{-7} M FMLP for 5 min. The cells were fixed in medium devoid of acetone and treated as above. (D) PMN were treated as in (C) but fixed in acetone.

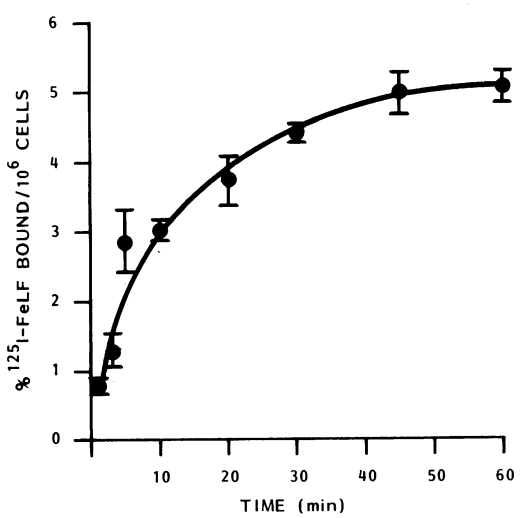

FIGURE 2 Time course of human iron-saturated LF binding to human PMN. PMN were added to the assay medium containing 25 nM ^{125}I-FeLF (sp act was 63 mCi/mg) and 250 nM cold LF at zero time and incubated at 4°C. The reaction was stopped by adding cold PBS/BSA to each tube at the indicated time intervals for assay of iron-saturated LF bound.

could also be identified about the perimeter in some cells fixed in acetone (Fig. 1d). These activated cells readily formed aggregates and appeared larger than the nonactivated cells. Cytochalasin B was used to facilitate optimal visualization of the immunoperoxidase product. Appropriate controls with nonimmune serum failed to demonstrate any reaction product.

Because LF appeared to be translocated to the surface of the cells during PMN activation, we sought to identify specific receptors on the PMN plasma membrane. Human PMN were incubated at 4°C with 25 mM Fe-saturated-LF in PBS containing 0.2% BSA. At various time intervals, the reaction was stopped by diluting the reagents with buffer. After 45 min, the binding curve tended to flatten (Fig. 2). Binding was displacable because washed cells previously incubated with 25 nM ^{125}I-FeLF for 45 min and resuspended in 0.1 ml PBS-BSA released ~75% of bound radioactivity into the medium when incubated for 10 min at 4°C with unlabeled FeLF at 10-fold excess. On the other hand, in a series of three experiments comparable concentrations of transferrin or IgG failed to dissociate ^{125}I-FeLF.

In a series of three experiments the specific binding

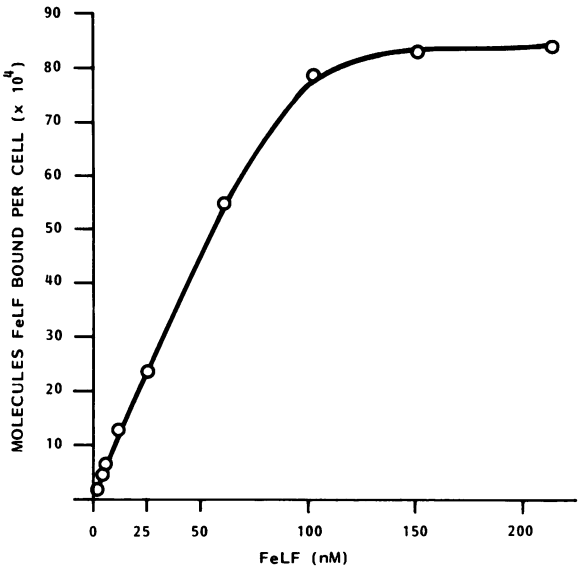

FIGURE 3 Equilibrium binding curve for human FeLF binding to human PMN. 0.10 ml of 1×10^7 cells/ml was mixed with various amounts of iron-saturated LF for each binding assay. For each tube containing ^{125}I-FeLF, a control tube containing 10-fold excess unlabeled FeLF was added to assess nonspecific binding.

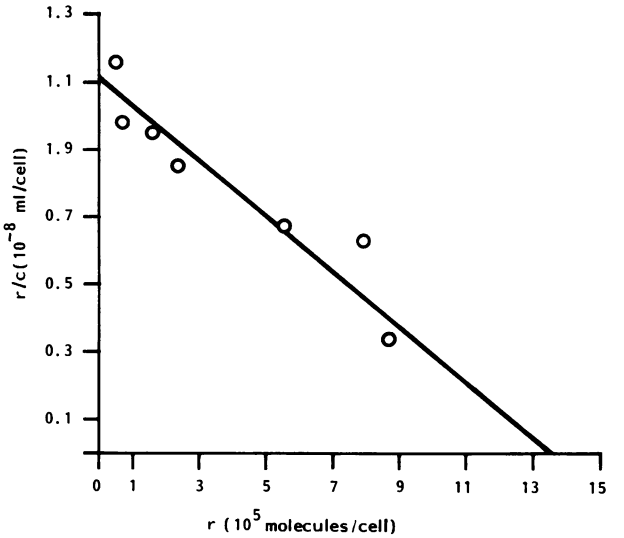

FIGURE 4 Scatchard plots of equilibrium binding. Values for specific binding to PMN were plotted according to Scatchard by using linear regression to find the line that best fit the experimental values. The correlation coefficient (r^2) for Fe-saturated-LF was 0.83, and the slope of the line represents the effective K_a, which was 5.2×10^6 liter/mol. Each iron-saturated LF was run in triplicate and the experiment was repeated twice. $n = 1.35 \times 10^6$ molecules/cell; $K_a = 5.2 \times 10^6$ liter/mole.

of Fe-saturated-LF was assessed by adding increasing amounts of the protein to PMN. After incubation at 4°C for 45 min, repeated centrifugation and washing, the radioactivity was measured in the pellet. PMN were saturated with FeLF when its concentration in the medium reached on the average 100 nM (Fig. 3).

Fig. 4 shows a plot of the binding date of Fig. 3 according to Scatchard. Plots of bound LF/free LF vs. the concentration of bound LF were linear indicating a single high-affinity binding site. The association constant (K_a) was found to be 5.2×10^6 M^{-1}. The number of binding sites per cell for FeLF was $\sim 1.35 \times 10^6$ sites/cell.

Since translocation of LF from the specific granules to the cell surface has been associated with aggregation, we wished to determine whether these responses could be accounted for by a reduction in negative cell surface charge. To quantitate relative changes in PMN surface charge the partitioning of the spin label CAT_{12} was measured. CAT_{12} bound to the PMN membrane gave a three-line first-derivative absorption spectrum with broad line-widths, which was easily resolved from the narrow line-width signal from the CAT_{12} remaining free in solution (Fig. 5). The low-field line-height of the bound signal ($h_{+1}{}^b$) and the high-field line-height of the free signal ($h_{-1}{}^f$) are proportional to the number of molecules in each environment. Changes in the partitioning after addition of exogenous factors were quantitated using Eq. 1.

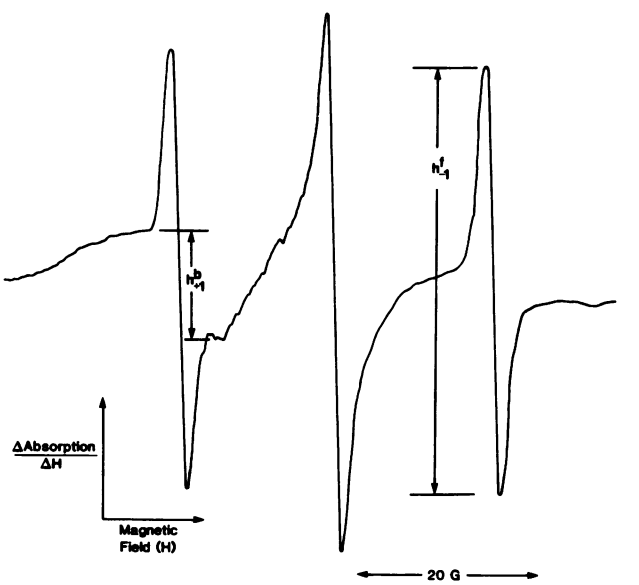

FIGURE 5 First derivative ESR absorption spectrum for CAT_{12} partitioned into intact PMN at 25°C. The indicated line-heights are a measure of the number of molecules free in solution ($h_{-1}{}^f$) and bound to the PMN membranes ($h_{+1}{}^b$) and are used with Eq. 1 to quantitate relative changes in cell surface potential. The magnetic field is measured in gauss.

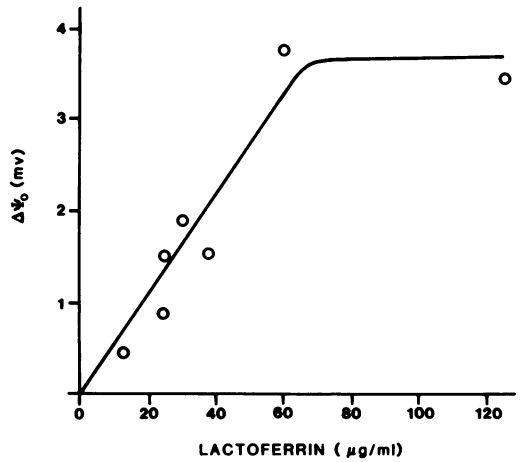

FIGURE 6 The relative reduction in surface potential in response to increasing concentrations of LF as determined by the partitioning of 30 μM CAT_{12} into 5.5 × 10^7 PMN/ml. Each point represents the mean of 5–10 samples. The effect of 60 and 120 μg/ml LF is significant at the $P < 0.025$ and $P < 0.01$ levels, respectively.

Addition of rabbit LF to samples containing 5.5 × 10^7 PMN/ml reduced the PMN surface potential in a dose-dependent manner (Fig. 6). The changes were measured relative to transferrin and buffer controls, which were indistinguishable from each other. The extent of the change was ~4 mV. However, a 10-min exposure to 10^{-7} M FMLP, a chemotactic factor known to alter PMN surface characteristics (32), resulted in a maximal reduction of ~15 mV in PMN surface potential (data not shown). From these data it appears that, in addition to LF, other factors or mechanisms also contribute to an alteration of the surface potential of activated PMN.

The amount of exogenous LF necessary to cause a change in surface potential can be compared to the number of LF receptors on the cell surface and to the amount of LF released from specific granules upon activation. Approximately 6% of the total cellular LF is released when PMN are stimulated with 10^{-7} M FMLP (11). This amounts to 0.6 μg/10^6 PMN or 4.6 × 10^6 molecules of LF/PMN. Although the amount remaining bound under these conditions is not known, there appears to be sufficient LF present to saturate the 1.35 × 10^6 receptors/cell and, in addition, to occupy nonspecific binding sites. When 6 × 10^6 molecules of LF are added per PMN, the receptors appear fully saturated (Fig. 3) and the ratio of specific to nonspecific binding is ~1:1. Thus, the total number of LF molecules bound to the surface could be as high as 2.7 × 10^6 molecules/PMN. In the ESR studies, addition of exogenous LF at 8 × 10^6 molecules per cell produced a 4-mV reduction in surface potential. If approximately half of the added LF is again bound to the cell surface, the resulting 4 × 10^6 molecules bound per PMN is in close agreement with the known total binding at a similar concentration (2.7 × 10^6 molecules/PMN) and the amount of LF released upon stimulation with FMLP (4.6 × 10^6 molecules/PMN). Thus, it appears that the observed change in surface potential can be accounted for by LF released from specific granules.

Since binding of LF to the PMN is associated with PMN-to-PMN interaction, we sought to identify whether LF-coated PMN could promote PMN-macrophage interactions. The LF-coated PMN were recognized and subsequently internalized into a macrophage phagosome more readily than PMN coated with another specific granule constituent, lysozyme, as seen in Table I. Also, in contrast to lysozyme, LF promoted in a dose-dependent fashion the uptake of PMN into macrophages. Although LF promoted uptake of PMN by macrophages, neither exogenous LF nor lysozyme altered the ability of macrophages to ingest C3-coated lipopolysaccharide-paraffin oil droplets (control, 0.48±0.03 mg oil/10^7 macrophages/10 min; 12 μg/ml added LF, 0.50±0.01; 12 μg/ml added lysozyme 0.50±0.01).

DISCUSSION

When PMN encounter chemotactic stimuli in vitro their adherence to vascular endothelium is enhanced (32, 33). If the PMN are flowing in the vasculature under similar circumstances they will form leukoaggregates. Concomitantly, chemotactic factors induce

TABLE I
Effect of Lactoferrin on Macrophage Ingestion of Polymorphonuclear Leukocytes

Dose		% Macrophage ingesting PMN	Mean number PMN/10 macrophages
	μg/ml		
LF	3	9.0	0.7
Lysozyme	3	11.0	0.9
LF	6	26.5	1.7
Lysozyme	6	11.9	1.1
LF	12	26.5	2.7
Lysozyme	12	10.4	0.7

Rabbit PMN leukocytes were incubated with either rabbit LF or lysozyme for 15 min at 37°C. Rabbit alveolar macrophages were then added to a suspension of treated PMN at a ratio 1:3. Each data point represents at least 100 macrophages counted. *P* values were calculated based on studentized range after an angular transformation of the percentages that indicated that LF was significantly different ($P < 0.01$) than lysozyme in promoting ingestion (51).

a transient neutropenia in animals (3–6). Similarly human and rabbit LF, a product of the specific granules, will promote adherence to endothelium and PMN aggregation as well as trigger a transient neutropenia (11, 12). Not only will chemotactic factors promote PMN stickiness but direct encounter with opsonized bacteria will occasionally result in increased PMN stickiness (33, 34). Under the latter circumstance, LF has been demonstrated to be localized to the surface of the PMN independent of nascent phagosomes. In this study, our method of fixation was able to distinguish surface from intracellular binding of LF antibody. Activated PMN were fixed in formalin, stained with an immunoperoxidase conjugate and an antibody specific for LF. With this approach it was possible to identify LF at the surface of the PMN (35). In contrast, fixation of PMN with formalin-acetone allowed for penetration of the antibody-complex and identification of LF in the cytosol. Granule surface staining for LF appeared in ~70% of the cells. Our observation is consistent with the finding that not all PMN achieve the same state of heightened activation (36).

The synthetic chemotactic peptide FMLP has proven to be a useful probe in characterizing the events leading to PMN activation. The synthesis of radioactively labeled oligopeptides of high specific activity with the same biological potency as the unlabeled peptides has allowed studies of the binding of the peptide to the surface of the PMN (37). With the use of tritiated peptides, it was shown by direct binding techniques that human PMN have specific high-affinity receptors for the oligopeptides (38). The equilibrium dissociation constant (K_d) for the ligand is ~20 nM. Human PMN have ~50,000 receptors per cell. Similarly, LF was found to bind to PMN at 4°C with saturation kinetics suggesting specific receptor activity. Binding studies were conducted at ice-bath temperature to inhibit PMN aggregation, which may contribute to nonspecific trapping of LF (39) and to prevent internalization of bound LF in PMN similar to that observed for a chemotactic peptide receptor (40). Scatchard analysis revealed a K_a for LF of $5.2 \times 10^6/M^{-1}$ and 1.35×10^6 receptor sites per cell. Others have shown that peritoneal macrophages will bind $\sim 20 \times 10^6$ LF molecules per cell with a K_d of 1 mM (41). Transferrin, another plasma iron bearing glycoprotein, has been shown to bind to reticulocytes (42), choriocarcinoma cells (43), and lymphoblastoid cell lines (44), but not to macrophages and PMN (41). The present study extends these observations; LF will bind not only to macrophages, but also to PMN. Binding of LF to macrophages inhibits the release of colony-stimulating activity (45). In an analogous fashion, LF can modulate the functional activities of the PMN. It serves to enhance PMN stickiness and also plays a role in hydroxyl radical formation by the activated cell (11, 12, 46). Potentially, the release of LF from effete PMN could in turn provide a mechanism of clearance of these cells by scavenger macrophages (47). In addition, this could arm the macrophage with PMN products such as the lysozymal enzymes myeloperoxidase and LF; both proteins are known to mediate and amplify the microbicidal capacity of H_2O_2 produced in the oxidative metabolic burst that accompanies phagocytosis.

Contact and communication between the PMN and its environment or other cells is made through the cell surface. The PMN, like most other cells, exhibits a negative resting cell surface charge (32). This charge tends to promote cell separation through electrostatic repulsion and thus aids in keeping the PMN free in suspension. Upon stimulation the PMN cell surface is altered such that the surface becomes more positive (less negative) (32). Thus, the tendency to remain separate is diminished and the tendency to become sticky is increased. In this study the change in the cell surface potential, which is related to surface change density by the Gouy-Chapman theory (22), was measured by determining the change in the partition coefficient for a charged probe molecule. The partition coefficient of this charged nitroxide spin probe was quantitated through the use of ESR. Addition of the positively charged protein LF (41) caused a relative change of 4 mV in the surface potential of the PMN; this is approximately one-third that observed after stimulation of the PMN with 10^{-7} M FMLP (unpublished observation). The implication of these results is that upon activation of the PMN, specific granule membrane products are recruited to the surface of the cell that increase the surface roughness, cell size, and diminish the negative potential (32, 48). These changes would increase the frictional drag, as previously reported in whole cell electrophoresis studies, and promote the attachment of PMN to other cellular surfaces.

Two patients with LF deficiency have been reported (49, 50). Abnormalities accompanying the observed deficiency of LF in the two patients included chemotaxis and respiratory burst activities. One patient's PMN failed to maintain sustained aggregation (49), whereas the other patient's cell did (50). When examined the former patient's PMN failed to adhere normally to endothelium upon stimulation with FMLP and reduce the negative surface charge to the same extent as control. Thus, the association of LF deficiency with these abnormalities further suggest that specific-granule products play a part in modulating PMN function.

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