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Surface Expression of Lactoferrin by Resting Neutrophils

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We examined the surface expression of lactoferrin by human neutrophils. Western blot analysis with anti-lactoferrin antibodies demonstrated the presence of a 78- to 79-kDa band in plasma membranes isolated from resting neutrophils that corresponded to the 78- to 79-kDa protein in neutrophil secondary granules. Flow cytometry using FITC-conjugated anti-lactoferrin antibodies confirmed that lactoferrin is expressed on the neutrophil surface. Preincubating the neutrophils in acidic (pH 3.9) buffer did not alter staining of the cells by the antibodies. Surface expression of lactoferrin was also detected on neutrophils in whole blood. Neutrophil activation by C5a or the calcium ionophore A23187 did not increase the surface expression of lactoferrin. Instead, the level of lactoferrin expression detected with one of two monoclonal antibodies was diminished after neutrophil activation, suggesting a possible conformational change in the lactoferrin. The surface-expressed lactoferrin may provide a mechanism for the interaction between lactoferrin-binding microorganisms and neutrophils.

Key Words: **neutrophil; lactoferrin; plasma membrane; secondary granule; cell activation.**

Lactoferrin is a 77- to 80-kDa glycoprotein synthesized by glandular epithelial cells and neutrophils (1, 2). Lactoferrin possesses a number of properties that, along with its sites of origin, indicate an important role for it in innate immunity, particularly along mucosal surfaces, and in the host defense function of neutrophils. Specifically, lactoferrin inhibits bacterial growth (1, 3), is directly cytotoxic for bacteria (4–6), and inhibits bacterial adherence (7) and colonization (8) of mucosal surfaces.

In neutrophils, lactoferrin is a distinguishing component of secondary (specific) granules (2), and release of lactoferrin is often used as an index for neutrophil activation at sites of inflammation. In the course of other studies ongoing in our laboratory, we obtained results suggesting that lactoferrin is also associated constitutively with neutrophil plasma membranes. The present study was performed to evaluate this conclusion.

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MATERIALS AND METHODS

Materials. Human milk lactoferrin was purchased from Sigma Chemical Co. (St. Louis, MO). Monoclonal antibodies generated against human neutrophil lactoferrin (AHN-9) (9) and human milk lactoferrin (2B8) were purchased from Pharmingen (San Diego, CA) and Biodesign International (Kennebunk, ME), respectively. Rabbit antiserum specific for human milk lactoferrin was purchased from Sigma Chemical Co., and the IgG component was isolated by Protein A–Sepharose chromatography (Pierce Chemical Co., Rockford, IL). Sources of additional antibodies were: FITC-conjugated, affinity-purified rabbit anti-human lactoferrin (Biodesign); FITC-conjugated goat anti-mouse IgG, FITC-conjugated goat anti-rabbit IgG, and FITC-conjugated mouse anti-human CD11b (Caltag Laboratories, Burlingame, CA); FITC-conjugated normal rabbit IgG (Sigma); and horseradish peroxidase-conjugated goat anti-mouse IgG and horseradish peroxidase-conjugated goat anti-rabbit IgG (Southern Biotechnology Associates, Birmingham, AL). Mouse IgG1 and rabbit IgG were purchased from Pharmingen and Sigma, respectively. Recombinant human C5a was purchased from Sigma, and A23187 and human serum albumin were purchased from Calbiochem (San Diego, CA).

Isolation of neutrophil plasma membranes and secondary granules. Neutrophils were isolated from venous blood of healthy adult donors by density gradient centrifugation as described previously (10). The cells were disrupted by nitrogen cavitation (350 psi for 20 min at 4°C) (11). After centrifugation at 500*g* (10 min at 4°C) to pellet unbroken cells and nuclei, plasma membrane and secondary (specific) granule fractions were isolated by centrifugation of the supernatant on a Percoll density gradient exactly as described elsewhere (11). The plasma membrane and granule fractions were suspended in 50 mM Mops (pH 7.2) and were stored in aliquots at −70°C until use. Plasma membranes were also isolated from HL-60 cells induced toward neutrophilic differentiation by culture for 5 days in 1.25% Me_2SO (12). The HL-60 cells were disrupted by nitrogen cavitation in disruption buffer (10 mM Hepes (pH 7.4) 0.34 M sucrose, 1 mM ATP, 2.5 mM $MgCl_2$, 0.1 mM PMSF, 2 μM antipain, and 2 μM pepstastin A) (13), and plasma membranes were isolated by centrifugation of the supernatant in a discontinuous (30%/50%) sucrose gradient (14). The plasma membranes were suspended in 50 mM Mops (pH 7.2) and were stored as described above for neutrophils. Protein concentrations were measured using the bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL).

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Western blot analysis. Plasma membranes, secondary granule fraction, or milk lactoferrin were boiled for 5 min in SDS sample buffer, and the proteins were separated by 8% SDS–PAGE in nonreducing conditions (15). The proteins were transferred electrophoretically to a Hybond-ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ), and nonspecific binding sites on the membrane were blocked by incubation in 3% BSA in Tris-buffered saline (pH 7.4) containing 0.1% Tween (TBST) for 1 h at room temperature. The membrane then was incubated with 0.1 μg/ml anti-lactoferrin antibody or control IgG in BSA–TBST for 1 h at room temperature. The membrane was washed three times (10 min each) in TBST and was incubated with a 1:6000 dilution (in BSA–TBST) of horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG for 1 h at room temperature. The membrane was washed as above, and bound antibody was visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech) using X-Omat film (Eastman Kodak, Rochester, NY).

Flow cytometric analysis of isolated neutrophils. Neutrophils were isolated as described above, and the neutrophils were treated or activated as described in the text. The cells were collected by centrifugation at 300*g* (5 min, 4°C), and 10^6 cells were incubated with 1.5 μg AHN-9 or 2B8, or mouse IgG1 control, in 25 μl PBS (pH 7.2) containing 0.1% gelatin and 0.1% azide (PBS-gel-azide) for 30 min on ice. The cells were washed twice in ice-cold PBS-gel-azide and then were incubated with 1 μg FITC-conjugated goat anti-mouse IgG in 25 μl PBS-gel-azide for 30 min on ice. The cells were washed twice in ice-cold PBS-gel-azide and were resuspended in the same buffer containing 1% formaldehyde for flow cytometric analysis. Fluorescence intensity of 10,000 cells in each sample was measured using a FACScan (Becton–Dickinson, San Jose, CA) instrument.

Flow cytometric analysis of whole blood. Blood was drawn into a EDTA-containing vacutainer tube (Becton–Dickinson), and 100 μl aliquots were incubated with 3 μg FITC-conjugated polyclonal anti-lactoferrin or 3 μg FITC-conjugated control rabbit IgG for 15 min on ice. Red cells then were lysed by addition of 2 ml FacsLyse (Becton–Dickinson) and incubation of the samples for 10 min on ice. The leukocyte pellet was collected by centrifugation at 300*g* for 5 min at 4°C and was washed twice with ice-cold PBS-gel-azide. The cells were resuspended in PBS-gel-azide containing 1% formaldehyde, and cell fluorescence was measured as described above.

RESULTS

Detection of Lactoferrin in Neutrophil Plasma Membranes

Neutrophil plasma membrane and secondary granule fractions were subjected to SDS–PAGE, and Western blot analysis was performed using two monoclonal antibodies, AHN-9 (9) and 2B8, and a polyclonal antibody specific for lactoferrin. Each of the three antibodies detected a major 78- to 79-kDa protein band in the plasma membrane fraction that comigrated with the major band in secondary granules and between the major 78-kDa band and minor 80-kDa band detected in milk lactoferrin (Fig. 1). All three antibodies also detected a less prominent band of approximately 85 kDa in the plasma membrane and specific granule fractions. Several low-molecular-weight bands of varying intensity were also observed in the specific granule fraction when the Western blot analysis was performed with the monoclonal antibody AHN-9 or with the polyclonal antibody. No protein bands were observed when mouse IgG1 or rabbit IgG was substituted for AHN9 and 2B9 or for the polyclonal antibody, respectively (Fig. 1). Additional experiments using the polyclonal anti-lactoferrin antibody demonstrated that the 78- to 79-kDa protein was only faintly detectable in plasma membranes isolated from Me_2SO-induced HL-60 cells (results not shown).

Surface Expression of Lactoferrin by Resting Neutrophils

Incubating neutrophils with monoclonal antibodies AHN-9 or 2B8, and subsequently with FITC-conjugated goat anti-mouse IgG, demonstrated that lactoferrin could be detected on the surface of resting neutrophils (Fig. 2). Both AHN-9 and 2B8 reacted with the neutrophils, with AHN-9 producing a stronger staining pattern. To determine if the surface-expressed lactoferrin might simply reflect membrane-bound lactoferrin, staining was also done after preincubating (5 min, 4°C) the neutrophils in buffer containing 10 mM lactic acid (pH 3.9), which dissociates IgE from high affinity $Fc_\varepsilon RI$ receptors (16). As shown in Fig. 2, the lactic acid pretreatment did not alter neutrophil reactivity with either AHN-9 or 2B8. Preincubating the neutrophils in 0.25 M NaCl or in buffer containing 10 mM EDTA likewise did not change level of lactoferrin surface expression (results not shown). Similar results were obtained for each pretreatment in three additional experiments.

Surface Expression of Lactoferrin by Neutrophils in Whole Blood

Neutrophil expression of CD11b/CD18, which is also associated with neutrophil specific granules (2), can increase, in some cases, as a consequence of the cell isolation procedure (17). Therefore, surface expression of lactoferrin by neutrophils in whole blood was evaluated to determine if the surface-expressed lactoferrin resulted from the neutrophil isolation procedure. As shown in Fig. 3, incubation with FITC-conjugated polyclonal anti-lactoferrin antibody yielded positive staining of the granulocyte population in whole blood. In contrast, no positive staining was observed for the monocyte or lymphocyte populations. The granulocyte, monocyte, and lymphocyte populations in the whole blood were identified based on prior staining with lineage-specific antibodies and by their characteristic light scatter properties. Similar results were obtained in two additional experiments.

Surface Expression of Lactoferrin on Activated Neutrophils

To assess whether neutrophil activation increased the surface expression of lactoferrin, neutrophils were stimulated with 10 nM C5a or 0.1 μg/ml calcium ionophore A23187 for 15 min prior to incubation with

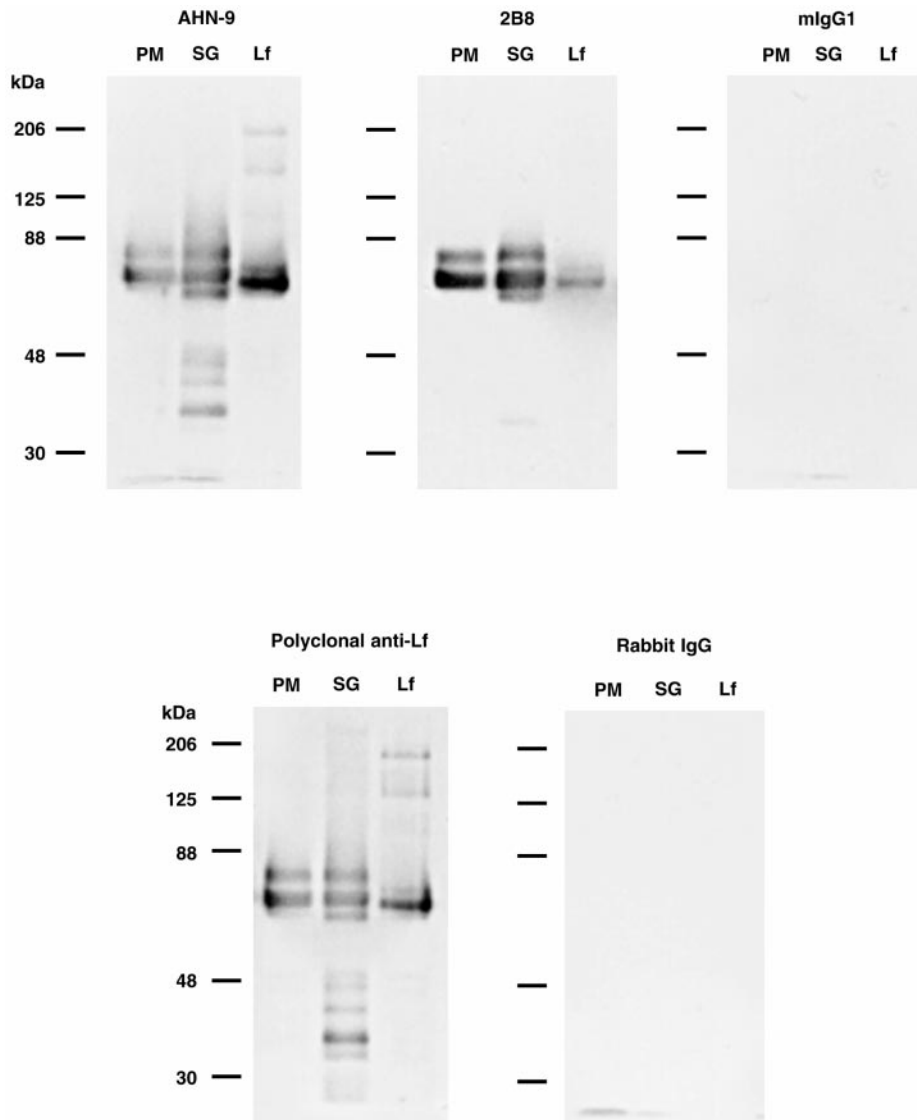

FIG. 1. Presence of lactoferrin in neutrophil plasma membranes. Neutrophil plasma membranes (PM) (10 μg), neutrophil secondary granules (SG) (2 μg), and 0.25 μg human milk lactoferrin (Lf) were subjected to SDS–PAGE, and the proteins were transferred to Hybond-ECL nitrocellulose. Western blot analysis was performed with the lactoferrin-specific monoclonal antibodies AHN-9 or 2B8, affinity purified rabbit anti-human lactoferrin antibody, control mouse IgG1, or control rabbit IgG as indicated. The blots were developed by enhanced chemiluminescence after incubation with horseradish peroxidase-conjugated secondary antibody as described under Materials and Methods. All the blots shown are from the same experiment.

monoclonal antibodies AHN-9 or 2B8. Cells were also incubated in buffer alone at 4°C or at 37°C as controls. The results in Fig. 4 show that neutrophil reactivity with AHN-9 diminished after stimulation by C5a or the calcium ionophore A23187, whereas reactivity with 2B8 was not altered. In the same experiment, incubation with C5a or A23187 increased expression of CD11b, thus confirming neutrophil activation under the experimental conditions. Similar results were obtained in four additional experiments. In the five experiments, the mean channel fluorescence of the AHN-9 positive population was significantly ($P < 0.05$) diminished by 49 ± 5% (mean ± SE) after activation of the neutrophils by 10 nM C5a. Conversely, the mean channel fluorescence of the CD11b positive cell population, which was examined in three of the five experiments, was significantly ($P < 0.05$) increased by 165 ± 45% after incubation with C5a.

DISCUSSION

These results demonstrate that lactoferrin, which is a characteristic marker of neutrophil secondary granules (2), is also expressed on the surface of intact resting neutrophils. Western blot analysis using two monoclonal antibodies as well as a polyclonal antibody

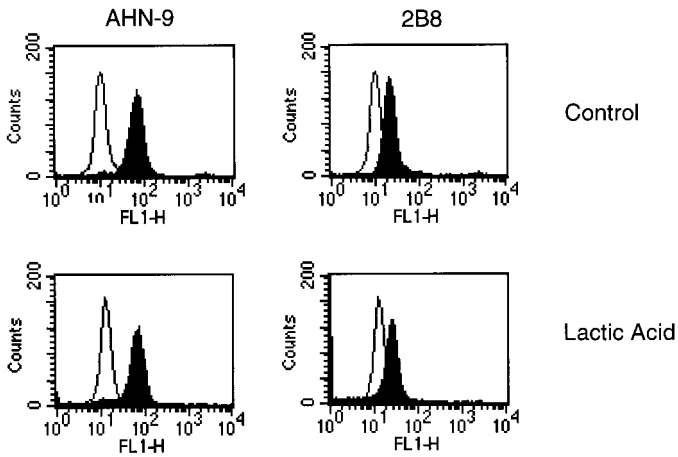

FIG. 2. Lactoferrin expression by resting neutrophils. Neutrophils (10^6) were preincubated in Hepes-albumin-glucose buffer (pH 7.4) (10) (Control) or in buffer comprised of 130 mM NaCl, 5 mM KCl, and 10 mM lactic acid (pH 3.9) (16) (Lactic acid) for 5 min at 4°C. The cells then were incubated in PBS-gel-azide with monoclonal antibody AHN-9 or 2B8, or mouse IgG1 control, and subsequently with FITC-conjugated goat anti-mouse IgG as described in the text. Mouse IgG1 (unfilled peak). AHN-9 or 2B8 (filled peak).

specific for human lactoferrin showed that surface expression of the lactoferrin correlates with the presence of a prominent 78–79 kDa band in plasma membranes that co-migrates with the major 78- to 79-kDa protein detected in secondary granules. Consistent with the deficiency of lactoferrin and other secondary granule proteins in differentiated HL-60 cells (2, 18), the 78- to 79-kDa protein was only minimally detectable in plasma membranes isolated from Me_2SO-induced HL-60 cells. These findings differ somewhat from those of another laboratory, which reported surface expression of lactoferrin by neutrophils only after activation or after isolation using dextran sedimentation (19). Different anti-lactoferrin antibodies were used in the previous study (19), but otherwise no explanation for the contrasting findings is immediately apparent. Of interest, surface expression by resting neutrophils of another secondary granule protein, the complement

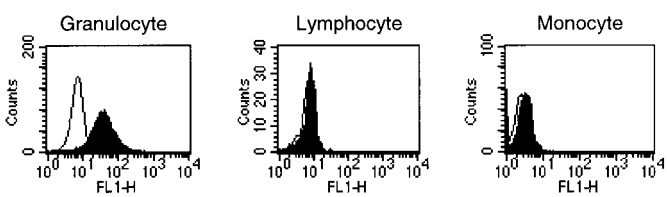

FIG. 3. Lactoferrin expression by neutrophils in whole blood. Whole blood (100 μl) was incubated with FITC-conjugated affinity-purified rabbit anti-human lactoferrin or FITC-conjugated control rabbit IgG for 15 min on ice as described under Materials and Methods. The red cells were lysed as described in the text, and fluorescence was measured by flow cytometry as described for Fig. 2. FITC-IgG control (unfilled peak). FITC-anti-lactoferrin (filled peak).

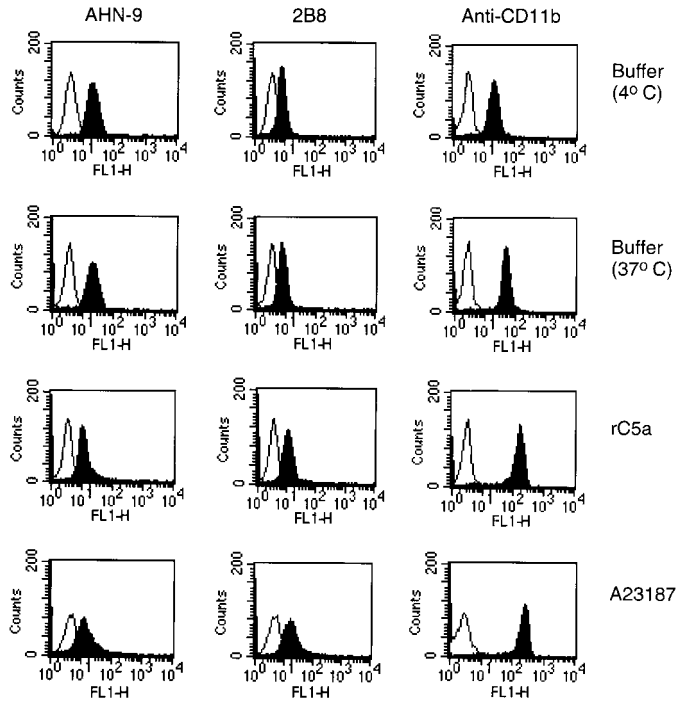

FIG. 4. Lactoferrin expression by activated neutrophils. Neutrophils (10^6/ml) were incubated in Hepes-albumin-glucose buffer (pH 7.4) containing 0.6 mM $CaCl_2$ and 1 mM $MgCl_2$ at 4°C or 37°C, or in the buffer (37°C) containing 10 nM C5a or 0.1 μg/ml A23187 for 15 min. The cells were washed twice with PBS-gel-azide and were incubated with monoclonal antibody AHN-9 or 2B8, mouse IgG1 control, or with FITC-conjugated anti-human CD11b as described in the text. Bound AHN-9 or 2B8 antibody was detected by subsequent incubation with FITC-conjugated goat anti-mouse IgG. The remainder of the staining protocol was performed as described for Fig. 2. Mouse IgG1 (unfilled peak). AHN-9, 2B8, or anti-CD11b (filled peak).

regulatory protein properdin, has been reported (20). As in the present study, surface expression of properdin was demonstrated by flow cytometry and was correlated with membrane localization of the properdin by Western blot analysis (20).

A possible explanation for the surface expression of lactoferrin is binding of lactoferrin to the plasma membrane during or subsequent to granule release. Indeed, lactoferrin receptors have been described for several cell types (1), although only a low affinity (K_d = 5 μM) receptor has been reported for neutrophils (21). Additionally, lactoferrin can also bind to surface glycosaminoglycans (22) or, as a basic protein (23), to other anionic constituents of the plasma membrane. The expression of elastase and cathepsin G, two cationic enzymes present in neutrophil primary granules (2), on the surface of activated neutrophils is attributed to a charge-dependent binding to the plasma membrane during their release (24–26). In contrast, however, to the increase in surface expression of cathepsin G and elastase after neutrophil activation (24–26), neutrophil activation by C5a or the calcium ionophore A23187 did not increase surface expression of lactoferrin in the

present study. Another laboratory (19) has reported increased expression of lactoferrin after neutrophil activation by phorbol myristate acetate or TNF-α, but, similar to the findings reported here, neutrophil activation by TNF-α or IL-8 did not alter surface expression of properdin (20). Although we cannot absolutely rule out the possibility of low level activation during the isolation procedure, the detection of lactoferrin on neutrophils in whole blood argues against low level activation as a basis for the expression. Of note, expression of the epitope recognized by monoclonal antibody AHN-9, but not that recognized by monoclonal antibody 2B8, is diminished after neutrophil activation by C5a or the calcium ionophore A23187. One interpretation of this finding is that neutrophil activation may induce a conformational change in the surface-associated lactoferrin.

If surface expression of lactoferrin does indeed reflect binding to the plasma membrane, the binding appears to be of relatively high affinity. First, preincubating neutrophils in buffer containing 10 mM lactic acid (pH 3.9), which readily dissociates IgE from high affinity $Fc_{\varepsilon}RI$ receptors (16), did not diminish the level of neutrophil staining by either of the two anti-lactoferrin monoclonal antibodies. Second, lactoferrin was also detected on neutrophils in whole blood, even though the concentration of lactoferrin in blood is less than 1 μg/ml ($<$12 nM) (27). It may be worth noting in this context that Western blot analysis with each of the three anti-lactoferrin antibodies also detected an approximately 85-kDa protein in the plasma membrane and secondary granule fractions. Lactoferrin is encoded by a single gene (28), and comparison of a partial cDNA sequence for neutrophil lactoferrin to the same sequence in milk lactoferrin indicates near identity for the two proteins (29, 30). The limited degree of heterogeneity (77 and 80 kDa) that is observed in the M_r of milk lactoferrin arises from differences in the degree of N-linked glycosylation (31). However, a variant arising from alternative mRNA splicing of the first exon has been described, albeit not in leukocytes (32). Thus, the possibility exists that the 85-kDa protein represents a membrane-localized species of lactoferrin arising from alternative mRNA splicing or possibly from differential post-translational processing.

The surface association of enzymatically active properdin and cathepsin G is postulated to contribute to neutrophil host defense functions (20, 25). Several findings suggest that surface-expressed lactoferrin may likewise contribute to neutrophil host defense function. Lactoferrin stimulates an increase neutrophil adhesion (21), one consequence of which is facilitated uptake of the neutrophils by macrophages (21). More recently, it has been reported that incubation with anti-lactoferrin antibody enhances neutrophil adherence to fibronectin and laminin as well as stimulates superoxide anion release (33). Antibodies against elastase or myeloperoxidase were without effect, and the authors postulated that a surface-expressed lactoferrin mediated the activation (33). Of particular interest in this context, several bacterial organisms, including *S. pneumoniae* (34), *N. gonorrhoeae* (35), and *E. coli* (36), have been reported to express "receptors" for lactoferrin. The receptors are postulated to provide a mechanism for the organisms to extract Fe^{3+} from lactoferrin (35). It is attractive to speculate, however, that the surface-associated lactoferrin described here may also enhance interaction between neutrophils and lactoferrin-binding microorganisms and possibly contribute to neutrophil activation by the microorganisms.

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