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Immobilized Lactoferrin Is a Stimulus for Eosinophil Activation[1]

Larry L. Thomas,[2]* Wei Xu,* and Tamir T. Ardon†

Eosinophils are strongly implicated in the pathogenesis of asthma, particularly in damage to the airway epithelial lining. We examined the potential for lactoferrin, a multifunctional glycoprotein present in the airway surface liquid, to activate eosinophils. Incubating eosinophils in tissue culture wells pretreated with 1–100 μg/ml human lactoferrin stimulated concentration-dependent superoxide production by eosinophils. The same concentrations of immobilized transferrin were without effect. The potency of immobilized lactoferrin was approximately one-third that of immobilized secretory IgA in the same experiments. In contrast, immobilized lactoferrin did not stimulate neutrophil superoxide production. Eosinophils bound lactoferrin as determined by flow cytometry and by binding of ^{125}I-labeled lactoferrin. Transferrin did not block binding of ^{125}I-labeled lactoferrin. Soluble lactoferrin, however, did not activate the eosinophils and did not block superoxide production stimulated by immobilized lactoferrin. Immobilized lactoferrin also stimulated release of eosinophil-derived neurotoxin and low levels of leukotriene C4 production; the latter was significantly enhanced in the presence of 100 pg/ml GM-CSF. GM-CSF also enhanced superoxide production and eosinophil-derived neurotoxin release stimulated by the lower concentrations of immobilized lactoferrin. Pretreatment of the lactoferrin with peptide *N*-glycosidase F or addition of heparin or chondroitin sulfate to the incubation contents had no or only a minimal effect on the activity of immobilized lactoferrin. These results demonstrate that lactoferrin adherent to the surface epithelium may contribute to the activation of eosinophils that infiltrate the airway lumen in eosinophil-associated disorders such as asthma. ***The Journal of Immunology*****, 2002, 169: 993–999.**

Eosinophils are important effector cells in host defense against helminth infection and in the pathogenesis of a variety of inflammatory disorders, most notably allergic diseases such as asthma (1, 2). In particular, the local accumulation of eosinophils within tissues such as the lungs is a hallmark of allergic disorders, and the numerous pro-inflammatory mediators released by eosinophils are strongly implicated in the pathophysiological changes in asthma and other allergic inflammatory diseases (3). Accordingly, elucidation of the mechanisms responsible for eosinophil recruitment and activation is critical to the full understanding of eosinophil-associated disorders.

Lactoferrin is a 78- to 80-kDa glycoprotein synthesized by glandular epithelial cells and mature neutrophils (4, 5). Although frequently used as a marker for neutrophil degranulation at sites of inflammation, lactoferrin is also one of the more abundant proteins in the airway surface liquid covering the mucosal epithelium (6). Given its localization and its well-recognized bacteristatic and bactericidal properties (7–9), lactoferrin is postulated to contribute to the bacterial host defense function of neutrophils and to play a protective role against bacterial pathogens at the airway mucosa (9). It is now clear, however, that the biological actions of lactoferrin are not restricted to its bacteristatic and bactericidal properties. Indeed, a wide array of actions has been reported for lactoferrin (4, 10, 11), including stimulating neutrophil aggregation and adhesion (12, 13) and enhancing NK cell activity (14).

A hallmark of eosinophil-mediated inflammation in the lungs is damage of the airway epithelial lining (3). The damage to airway epithelium is attributed to the cytotoxic actions of eosinophil granule proteins such as major basic protein and to oxidants produced by the interaction of eosinophil peroxidase and hydrogen peroxide in the presence of bromine (3, 15). Secretory IgA is the prominent Ab class in mucosal secretions and is one of the most effective stimuli for eosinophil superoxide production and degranulation when immobilized on a nonphagocytosable surface (16, 17). This finding has demonstrated the potential for eosinophil activation to occur within the airway, a conclusion supported by the presence of eosinophil granule proteins in mucus plugs and along the mucosal epithelial surface in asthmatic airways (18). The present study was performed, therefore, to determine whether immobilized lactoferrin might also serve as a stimulus for eosinophil activation, given the relatively high concentrations of lactoferrin in the airway surface liquid.

Materials and Methods

Cell isolation

Neutrophils were isolated from venous blood of healthy adult volunteers by density gradient centrifugation through lymphocyte separation medium (BioWhittaker, Walkersville, MD) as described previously (19) with one modification. Isotonicity was restored following the brief hypotonic lysis steps by the addition of 2× concentrated HBSS (Life Technologies, Grand Island, NY; without Ca^{2+} and Mg^{2+}) containing 5 mM HEPES, pH 7.4. The cells were suspended in HEPES (10 mM)-buffered HBSS (with Ca^{2+} and Mg^{2+}), pH 7.4, containing 1 mg/ml human serum albumin (HSA[3]; Sigma-Aldrich, St. Louis, MO; HEPES-HBSS-HSA buffer). Neutrophil purity was routinely >95%, with eosinophils representing the remainder of the cells. Eosinophils were isolated from the neutrophil preparations by

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[3] Abbreviations used in this paper: HSA, human serum albumin; EDN, eosinophil-derived neurotoxin; PNGase F, peptide *N*-glycosidase F.

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negative selection (20) using anti-CD16 immunomagnetic beads as described by the manufacturer (Miltenyi Biotec, Auburn, CA). The eosinophils were suspended in HEPES-HBSS-HSA buffer. Eosinophil purity was routinely >95% as determined by counting Wright-stained cytospin preparations. In some experiments an aliquot of the neutrophil preparation was held on ice for later use.

Superoxide production

Superoxide production was measured essentially as described previously (17). Briefly, wells in a 96-well (flat-bottom) tissue culture plate (Corning, Corning, NY) were coated with human milk lactoferrin (Sigma-Aldrich) or human secretory IgA (ICN Biomedical, Aurora, OH) by incubation with 50 μl of the indicated concentrations of proteins in PBS overnight at 4°C. Nonspecific protein binding sites were blocked by subsequent incubation with 100 μl of 25 mg/ml HSA in PBS for 2 h at 37°C, and the tissue culture wells were washed twice with PBS before use. Aliquots (5×10^4 cells) of eosinophils or neutrophils were added to the wells and were incubated in HEPES-HBSS-HSA buffer containing 50 μM cytochrome *c* (Sigma-Aldrich) for 120 min at 37°C in a Ceres UV900HDi microplate reader (Bio-Tek Instruments, Winooski, VT). The total incubation volume was 0.2 ml. Absorbance at 550 nm was recorded at 15-min intervals, and superoxide production was calculated as described previously (17). Results are expressed as nanomoles of superoxide per 10^5 cells after subtraction of spontaneous production, which was measured in tissue culture wells coated only with HSA. GM-CSF (R&D Systems, Minneapolis, MN), porcine heparin (Sigma-Aldrich), or chondroitin sulfate C (Sigma-Aldrich) was added to the incubation mixtures in some experiments as indicated.

Degranulation

Eosinophils (2×10^5) were incubated in the presence and the absence of 100 pg/ml GM-CSF in RPMI 1640 containing 1 mg/ml HSA for 4 h at 37°C in 5% CO_2 in tissue culture wells precoated with lactoferrin or secretory IgA as described above. The total incubation volume was 0.2 ml. Reactions were stopped by centrifugation at $300 \times g$ for 5 min at 4°C, and supernatants were stored at −20°C until measurement of eosinophil-derived neurotoxin (EDN) content by specific ELISA (MBL International, Watertown, MA). Spontaneous release of EDN was determined with cells incubated in HSA-coated wells.

Leukotriene C_4 production

Eosinophils (2×10^5) were incubated in the presence and absence of 100 pg/ml GM-CSF in RPMI 1640 containing 10 mM HEPES for 1 h at 37°C in tissue culture wells precoated with lactoferrin or secretory IgA as described above, with one modification. The tissue culture wells were not treated with HSA, and HSA was not added to the incubation buffer to minimize the loss of leukotriene C_4. The total incubation volume was 0.2 ml. Reactions were stopped by centrifugation at $300 \times g$ for 5 min at 4°C, and supernatants were stored at −20°C until measurement of leukotriene C_4 content by a leukotriene C4/D4/E4 ELISA (Amersham Pharmacia Biotech, Piscataway, NJ). Spontaneous leukotriene C_4 production was determined with eosinophils incubated in untreated tissue culture wells.

Flow cytometry

Eosinophils (10^6 cells) were incubated with or without the indicated concentrations of lactoferrin in 100 μl HEPES-HBSS-HSA buffer for 90 min at 4°C. The cells were collected by centrifugation of the mixtures at $300 \times g$ for 5 min at 4°C, and the cells were incubated with 1.5 μg FITC-conjugated polyclonal anti-lactoferrin (Sigma-Aldrich) or FITC-conjugated rabbit IgG (Sigma-Aldrich) 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 were suspended in the same buffer containing 1% formaldehyde for analysis by flow cytometry. The fluorescence intensity of 10,000 cells in each sample was measured using a FACScan flow cytometer (BD Biosciences, San Jose, CA).

Binding of radiolabeled lactoferrin

Lactoferrin (100 μg) was radioiodinated using Iodogen iodination reagent (Pierce) according to the procedure supplied by the manufacturer. Lactoferrin was incubated with 400 μCi $Na^{125}I$ (Perkin-Elmer, Boston, MA) for 3 min, and ^{125}I-labeled lactoferrin was separated from free $Na^{125}I$ by chromatography through a 5-ml D-salt dextran desalting column (Pierce) using PBS as the elution buffer. The protein concentration of the ^{125}I-labeled lactoferrin was measured by bicinchoninic acid assay (Pierce). The sp. act. of the ^{125}I-labeled lactoferrin was 34,800 cpm/pmol. Immobilized ^{125}I-labeled lactoferrin (30 μg/ml) retained full ability to stimulate eosinophil superoxide production (data not shown). Binding of ^{125}I-labeled lactoferrin by eosinophils was determined using a modification of previously described protocols for eosinophils (17, 21). Eosinophils (2×10^6) were incubated with the indicated concentrations of ^{125}I-labeled lactoferrin alone and in the presence of excess unlabeled lactoferrin in RPMI 1640 containing 20 mM HEPES, 0.5% BSA, and 0.1% sodium azide (21) in siliconized glass tubes for 2 h at room temperature on a circular oscillating platform. In some experiments, as indicated, binding was measured in the presence of excess unlabeled transferrin. The total reaction volume was 0.15 ml. Reactions were stopped by centrifugation ($1300 \times g$ for 4 min) of the reaction mixture through 200 μl FCS in a 1.5-ml microcentrifuge tube. The supernatant was removed by careful aspiration, and after quick-freezing on dry ice the tip of the tube containing the cell pellet was excised, and the radioactivity was measured by gamma counting (γ 5500B; Beckman Coulter, Fullerton, CA). Specific binding was determined as the difference between total binding and binding in the presence of the excess unlabeled lactoferrin. Binding constants were determined by Scatchard analysis (22).

Deglycosylated lactoferrin

Lactoferrin (1 mg/ml) was incubated without or with 10^5 U/ml peptide *N*-glycosidase F (PNGase F; New England Biolabs, Beverly, MA) in PBS for 72 h at 37°C. The lactoferrin was stored in aliquots at −70°C. Deglycosylation was assessed by a reduction in the apparent M_r as determined in Coomassie blue-stained SDS-PAGE gels and by reactivity with HRP-conjugated Con A (EY Laboratories, San Mateo, CA). For Coomassie-stained gels, 10 μg protein was subjected to SDS-PAGE in 8% gels under nonreducing conditions (23). For reactivity with HRP-conjugated Con A, 0.2 μg protein was subjected to SDS-PAGE as described above and was transferred electrophoretically to Hybond ECL nitrocellulose (Amersham Pharmacia Biotech). After blocking with 3% gelatin in TBST, the membrane was incubated with 0.2 μg/ml HRP-conjugated Con A in TBST containing 3% gelatin for 1 h at room temperature. The blot was washed extensively with TBST, and positive bands were visualized by ECL.

Statistical analysis

Statistical analysis was performed using Student's paired *t* test. Statistical significance was set at $p < 0.05$.

Results

Immobilized lactoferrin stimulates eosinophil superoxide production

The capacity of immobilized lactoferrin to stimulate eosinophil superoxide production was examined by incubating eosinophils in tissue culture wells preincubated with 1–100 μg/ml lactoferrin overnight at 4°C. This concentration range corresponds to the concentrations of lactoferrin measured in airway surface liquid (6) and to the effective concentration range for stimulation of eosinophil superoxide production by immobilized secretory IgA (17). The results presented in Fig. 1*A* show that lactoferrin immobilized at concentrations of 10 μg/ml or greater stimulated marked superoxide production over the 2-h incubation period. After an ~15-min lag, superoxide production increased with time over the subsequent 45–60 min of incubation and then reached a plateau. Superoxide production stimulated by immobilized secretory IgA displayed a similar time course in the same experiment (Fig. 1*B*).

Plotting the level of superoxide production measured at the 2-h point in five experiments as a function of the concentration of immobilized lactoferrin or immobilized secretory IgA confirmed the concentration requirements illustrated in Fig. 1, *A* and *B*. Specifically, immobilized lactoferrin at concentrations <3 μg/ml did not stimulate superoxide production, whereas 30 μg/ml immobilized lactoferrin produced a maximum response of ~5 nmol superoxide/10^5 eosinophils (Fig. 1*C*). The addition of 100 pg/ml GM-CSF to the incubation mixture significantly enhanced the amount of superoxide production stimulated by 10 μg/ml immobilized lactoferrin (Fig. 1*C*), but GM-CSF did not increase the level of superoxide production stimulated by the higher concentrations of immobilized lactoferrin. Immobilized secretory IgA stimulated a concentration-dependent superoxide production over the same concentration range (Fig. 1*D*), as reported previously by

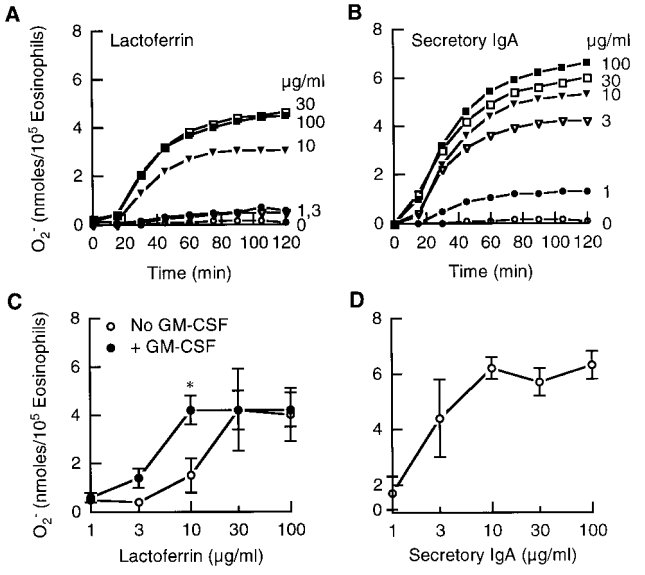

FIGURE 1. Stimulation of eosinophil superoxide (O_2^-) production by immobilized lactoferrin and immobilized secretory IgA. Eosinophils were incubated in tissue culture wells preincubated with the indicated concentrations of lactoferrin or secretory IgA as described in the text. Representative time courses from a single experiment are shown for O_2^- production stimulated by the indicated concentrations of immobilized lactoferrin (*A*) and immobilized secretory IgA (*B*). The concentration requirements for stimulation of O_2^- production by immobilized lactoferrin (*C*) and immobilized secretory IgA (*D*) determined at the 2-h point are shown as the mean ± SEM for five experiments, including the experiment shown in *A* and *B*. The values are corrected for spontaneous O_2^- production (0.7 ± 0.6 nmol $O_2^-/10^5$ eosinophils) and, in *C*, for GM-CSF (100 pg/ml) stimulated O_2^- production (2.8 ± 0.7 nmol $O_2^-/10^5$ eosinophils). *, $p < 0.05$ compared with the spontaneous value.

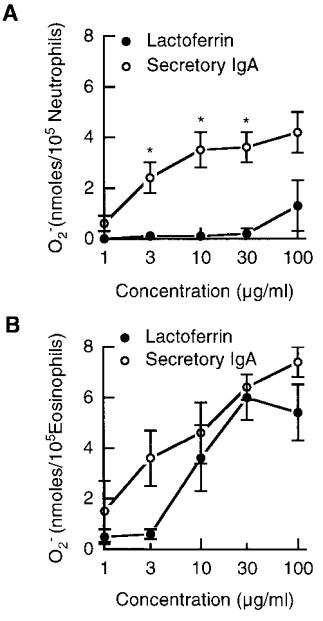

FIGURE 2. Comparison of neutrophil and eosinophil superoxide (O_2^-) production in response to immobilized lactoferrin and immobilized secretory IgA. Neutrophils (*A*) and eosinophils (*B*) isolated from the same individuals were incubated with the indicated concentrations of immobilized lactoferrin or secretory IgA as described for Fig. 1. Results are the mean ± SEM for four experiments after subtraction of the spontaneous values, which were <0.1 nmol/10^5 cells for both neutrophils and eosinophils. *, $p < 0.05$ compared with the value for immobilized secretory IgA.

others (17). The amount of superoxide production stimulated by immobilized secretory IgA in these experiments was ~50% greater than that stimulated by immobilized lactoferrin and peaked at the 10 µg/ml concentration (in the absence of GM-CSF).

In results not shown ($n = 4$), incubating eosinophils with suboptimal concentrations (3 or 10 µg/ml) of immobilized lactoferrin and suboptimal concentrations (1 or 3 µg/ml) of immobilized secretory IgA in combination resulted in additive levels of superoxide production. In additional results not shown ($n = 3$), incubating eosinophils with 1–100 µg/ml immobilized transferrin did not stimulate any superoxide production.

Immobilized lactoferrin does not stimulate neutrophil superoxide production

The ability of immobilized lactoferrin to also stimulate superoxide production by neutrophils was examined using neutrophils and eosinophils isolated from the same donors. Incubating neutrophils with 1–100 µg/ml immobilized lactoferrin for up to 2 h did not stimulate significant superoxide production (Fig. 2*A*). In contrast, the same concentrations of immobilized secretory IgA stimulated marked superoxide production by the neutrophils (Fig. 2*A*), with a time course (results not shown) and concentration dependence similar to those observed for eosinophil superoxide production in the same experiments (Fig. 2*B*). Only at the 100 µg/ml concentration did the level of neutrophil superoxide production stimulated by immobilized lactoferrin not differ significantly from that stimulated by immobilized secretory IgA. The amount of neutrophil superoxide production stimulated by 100 µg/ml immobilized lactoferrin, however, was only 25% the amount of eosinophil superoxide production (5.5 ± 1.4 nmol/10^5 eosinophils) stimulated by 30 µg/ml immobilized lactoferrin in the same experiments (Fig. 2*B*). In these experiments immobilized lactoferrin and immobilized secretory IgA stimulated similar levels of superoxide production by the eosinophils (Fig. 2*B*).

Lactoferrin binds to eosinophils

To confirm that eosinophils bind lactoferrin, eosinophils were incubated with or without 30 µg/ml soluble lactoferrin for 90 min at 4°C. The presence of bound lactoferrin then was determined by flow cytometry using FITC-conjugated IgG anti-human lactoferrin or FITC-conjugated normal rabbit IgG. In the absence of lactoferrin, FITC-conjugated anti-lactoferrin Ab did not display any specific reactivity with eosinophils (Fig. 3*A*). In contrast, incubating the eosinophils with 30 µg/ml lactoferrin produced a marked increase in the fluorescence intensity following reaction with the

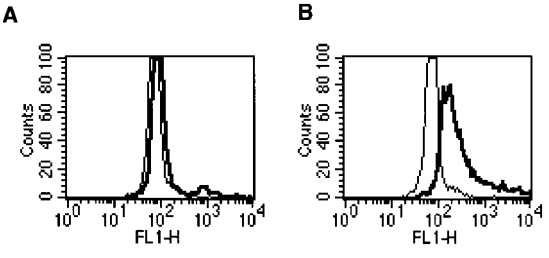

FIGURE 3. Flow cytometric analysis lactoferrin binding to eosinophils. Eosinophils were incubated in the absence (*A*) or the presence (*B*) of 30 µg/ml lactoferrin for 90 min at 4°C as described in the text. Bound lactoferrin was detected by flow cytometry after subsequently incubating the cells with 1.5 µg FITC-IgG anti-human lactoferrin (heavy line) or FITC-IgG (fine line) as described in the text. Similar results were obtained in two additional experiments.

FITC-conjugated anti-lactoferrin Ab (Fig. 3*B*). Incubating eosinophils with 100 μg/ml lactoferrin did not produce any further increase in the level of fluorescence intensity with the FITC-anti-lactoferrin Ab (results not shown).

Binding experiments using ^{125}I-labeled lactoferrin were performed to examine further the binding of lactoferrin by eosinophils. Incubating eosinophils with 23–180 nM ^{125}I-labeled lactoferrin alone and in the presence of 5 μM unlabeled lactoferrin for 2 h at room temperature confirmed specific binding of the ^{125}I-labeled lactoferrin (Fig. 4). Binding approached saturation and suggested the presence of two binding affinities. The limited concentration range and donor variability in the level of ^{125}I-labeled lactoferrin bound precluded precise determination of binding parameters. Nevertheless, analysis of binding data obtained in three experiments yielded two classes of receptors: one with a K_d of 47 ± 19 nM (mean ± SE) and comprising ~78,000 ± 13,000 molecules/eosinophil, and a second with a K_d of ~260 nM and comprising up to ~620,000 molecules/eosinophil. Eosinophil binding of ^{125}I-labeled lactoferrin (90 nM) was not inhibited in the presence of 5 μM transferrin ($n = 2$; results not shown).

Soluble lactoferrin does not influence eosinophil superoxide production

The capacity of soluble lactoferrin to stimulate eosinophil superoxide production was examined by incubating eosinophils with 1–100 μg/ml soluble lactoferrin for 2 h at 37°C in tissue culture wells coated only with HSA. The results presented in Fig. 5*A* show that soluble lactoferrin stimulated minimal superoxide production by eosinophils, whereas immobilized lactoferrin stimulated superoxide production in the expected concentration-dependent manner in the same experiments. Additional experiments demonstrated that addition of 1–100 μg/ml soluble lactoferrin did not inhibit eosinophil superoxide production stimulated by 30 μg/ml immobilized lactoferrin (Fig. 5*B*).

Immobilized lactoferrin stimulates eosinophil degranulation and leukotriene C_4 release

The ability of immobilized lactoferrin to stimulate eosinophil degranulation was assessed by EDN release after incubating eosinophils with 3–100 μg/ml immobilized lactoferrin for 4 h at 37°C in a 5% CO_2 atmosphere. The results presented in Fig. 6*A* show that immobilized lactoferrin stimulated the net release of up to ~1000 ng EDN/10^6 eosinophils in a concentration-dependent manner, with maximum release observed at the 100 μg/ml concentration. The addition of 100 pg/ml GM-CSF significantly enhanced EDN release stimulated by 3 and 10 μg/ml immobilized lactoferrin. In the same experiments immobilized secretory IgA stimulated the net release of ~500 ng/ml EDN at each of the concentrations tested over the range of 3–100 μg/ml (results not shown).

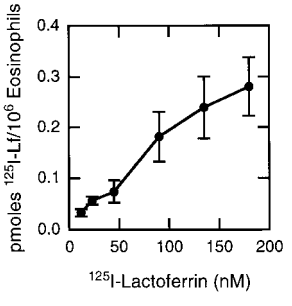

FIGURE 4. Binding of ^{125}I-labeled lactoferrin by eosinophils. Eosinophils were incubated with the indicated concentrations of ^{125}I-labeled-lactoferrin (Lf; 34,800 cpm/pmol) for 2 h at room temperature as described in the text. Specific binding is shown as the mean ± SEM of three to six experiments using eosinophils from five individuals. Specific binding was obtained by subtracting nonspecific binding measured in the presence of 5 μM unlabeled lactoferrin from total binding.

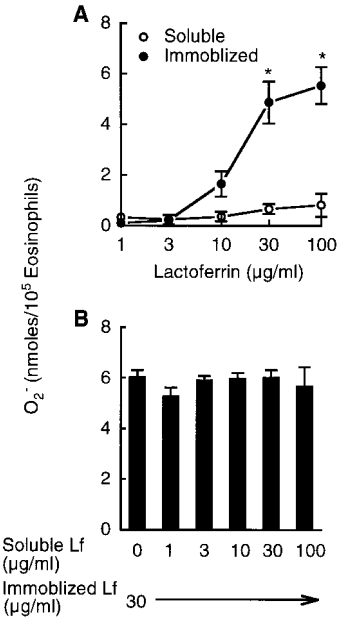

FIGURE 5. Effect of soluble lactoferrin on eosinophil superoxide (O_2^-) production. *A*, Eosinophils were incubated with the indicated concentrations of soluble or immobilized lactoferrin as described for Fig. 1. Results are the mean ± SEM for four experiments after subtraction of spontaneous production (<0.1 nmol/10^5 eosinophils). *, $p < 0.05$ compared with the value for immobilized lactoferrin. *B*, Eosinophils were incubated with 30 μg/ml immobilized lactoferrin in the presence of the indicated concentrations of soluble lactoferrin. Results are the mean ± SEM for three experiments after subtraction of the spontaneous value (0.2 ± 0.1 nmol/10^5 eosinophils).

The effect of immobilized lactoferrin on leukotriene C_4 production by eosinophils was evaluated in additional experiments. Incubating eosinophils with 3–100 μg/ml immobilized lactoferrin stimulated only low levels of leukotriene C_4 production over a 1-h incubation period (Fig. 6*B*). The addition of 100 pg/ml GM-CSF, however, significantly enhanced leukotriene C_4 release stimulated by 10–100 μg/ml immobilized lactoferrin. Whereas incubation with 10 μg/ml immobilized lactoferrrin alone and 100 pg/ml GM-CSF alone stimulated the production of 115 and 175 pg leukotriene C_4/10^6 eosinophils, respectively, incubation with the two stimuli together resulted in the production of 955 pg leukotriene C/10^6 eosinophils.

Eosinophil activation by immobilized lactoferrin is not mediated by N*-linked oligosaccharides or the glycosaminoglycan binding site in lactoferrin*

To assess whether the *N*-linked oligosaccharides in lactoferrin (24) contributed to eosinophil activation, the activities of lactoferrin deglycosylated by PNGase F treatment and lactoferrin treated in the same manner but in the absence of PNGase F (mock-deglycosylated lactoferrin) were compared. The results show that incubating eosinophils with 1–100 μg/ml immobilized deglycosylated lactoferrin stimulated superoxide production to the same extent and in the same concentration-dependent manner as immobilized mock-deglycosylated lactoferrin (Fig. 7*A*). Immobilized mock-deglycosylated lactoferrin produced the same response as immobilized control lactoferrin (results not shown). Deglycosylation of

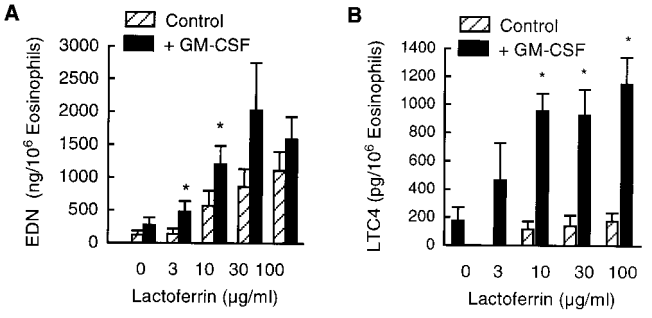

FIGURE 6. Stimulation of eosinophil EDN release and leukotriene C_4 (LTC4) release by immobilized lactoferrin. *A*, Eosinophils were incubated for 4 h (*A*) or 1 h (*B*) at 37°C in tissue culture wells preincubated with the indicated concentrations of lactoferrin in the absence and the presence of 100 pg/ml GM-CSF. EDN release (*A*) and leukotriene C_4 release (*B*) are shown as the mean ± SEM for five experiments and four experiments, respectively. *, $p < 0.05$ compared with the additive effects of immobilized lactoferrin and GM-CSF.

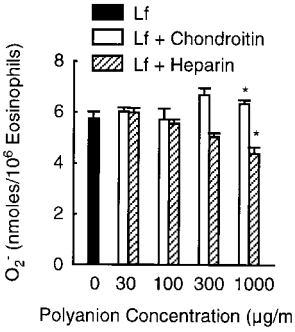

FIGURE 8. Effect of heparin and chondroitin sulfate on eosinophil superoxide (O_2^-) production stimulated by immobilized lactoferrin (Lf). Eosinophils were incubated in the absence or the presence of the indicated concentrations of heparin or chondroitin sulfate for 120 min at 37°C in tissue culture wells preincubated with 30 μg/ml lactoferrin. Results are the mean ± SEM for four experiments after subtraction of the spontaneous value (0.6 ± 0.4 nmol/10^5 eosinophils). *, $p < 0.05$ compared with control.

PNGase F-treated lactoferrin was confirmed by reduction in the M_r of the protein in SDS-PAGE (Fig. 7*B*) and by diminished reactivity with Con A (Fig. 7*C*).

The effects of heparin and chondroitin sulfate on eosinophil activation by immobilized lactoferrin were evaluated in three additional experiments to assess the involvement of the putative glycosaminoglycan binding site in lactoferrin (25, 26) in the response. The addition of 30–1000 μg/ml heparin inhibited superoxide production stimulated by 30 μg/ml immobilized lactoferrin by ~25% at the highest concentration tested (Fig. 8). Heparin had a similar effect on superoxide production stimulated by 10 μg/ml immobilized secretory IgA in the same experiments, with 1000 μg/ml heparin inhibiting the response by 28 ± 11% (results not shown). The inhibition, however, did not achieve statistical significance ($p = 0.07$). Chondroitin sulfate at 1000 μg/ml caused a slight, but statistically significant, increase in superoxide production stimulated by immobilized lactoferrin.

Discussion

Many of the effects of lactoferrin on immune and inflammatory cell function that have been described to date are inhibitory in nature, including the inhibition of several LPS-stimulated responses (10, 11). The results presented here demonstrate, however, that immobilized lactoferrin in concentrations similar to those present in airway surface liquid (6) is an effective stimulus for eosinophil superoxide production and degranulation as well as for low levels of leukotriene C_4 release. Eosinophil activation was triggered by lactoferrin that had been immobilized at concentrations >3 μg/ml, and the maximum or near-maximum response was observed in tissue culture wells that had been preincubated with 30 μg/ml lactoferrin. Similar to its effect on superoxide production and degranulation stimulated by other eosinophil stimuli (27–29), the presence of a low concentration (100 pg/ml) of GM-CSF significantly enhanced the level of eosinophil superoxide production and EDN release stimulated by immobilized lactoferrin. GM-CSF, however, enhanced the eosinophil responses stimulated only by the lower concentrations of immobilized lactoferrin. The net result of the GM-CSF presence, thus, was to reduce the concentration of immobilized lactoferrin required to stimulate maximal superoxide production or EDN release by ~3-fold, although this effect was most evident for superoxide production (Fig. 1*C*). GM-CSF also markedly enhanced eosinophil leukotriene C_4 production stimulated by concentrations of immobilized lactoferrin >3 μg/ml. In the absence of GM-CSF, immobilized lactoferrin stimulated levels of leukotriene C_4 release less than those reported previously for immobilized IgG (30, 31), but similar to levels reported for fMLP (32, 33). The level of leukotriene C_4 production stimulated by immobilized lactoferrin in the presence of GM-CSF

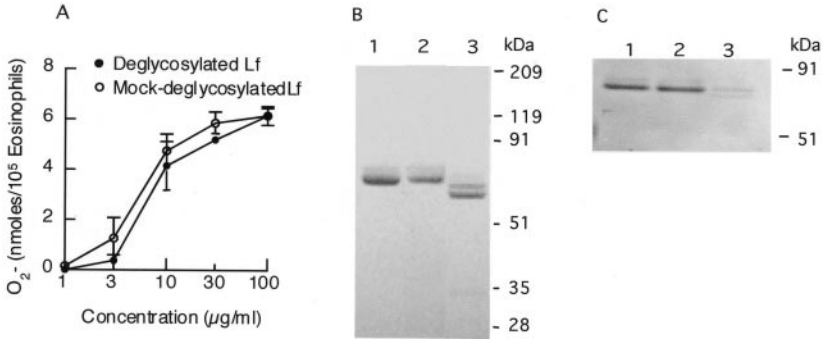

FIGURE 7. Stimulation of eosinophil superoxide (O_2^-) production by immobilized deglycosylated lactoferrin (Lf). *A*, Eosinophils were incubated for 120 min at 37°C in tissue culture wells preincubated with the indicated concentrations of deglycosylated lactoferrin or lactoferrin subjected to the same treatment as deglycosylated lactoferrin, but in the absence of PNGase F (mock-deglycosylated lactoferrin). Results are the mean ± SEM for four experiments after subtraction of the spontaneous value (0.2 ± 0.2 nmol/10^5 eosinophils). *B*, Coomassie-stained PAGE gel of 10 μg control lactoferrin (*lane 1*), mock-deglycosylated lactoferrin (*lane 2*), and deglycosylated lactoferrin (*lane 3*). *C*, Western blot of 0.2 μg control lactoferrin (*lane 1*), mock-deglycosylated lactoferrin (*lane 2*), and deglycosylated lactoferrin (*lane 3*) detected with HRP-conjugated Con A.

is similar to that obtained with fMLP for IL-5-primed eosinophils (32, 33). The eosinophil responses to immobilized lactoferrin, however, may be at least somewhat selective, as initial findings indicate that immobilized lactoferrin stimulates minimal or no IL-8 production.

Lactoferrin receptors have been described previously for a variety of cells, including various leukocytes (34–39) and epithelial cells (40). The binding affinities reported for the different cells vary widely, with the dissociation constants ranging from nanomolar to micromolar concentrations (34, 35, 37–39, 41). The results presented here for binding of ^{125}I-labeled lactoferrin by the eosinophils indicate that the affinity of the lactoferrin receptor on eosinophils also falls within this range. Indeed, the results suggest that eosinophils possess two classes of lactoferrin receptors, with dissociation constants of ~47 and 260 nM. Two classes of lactoferrin receptors have also been reported for the human promonocyte THP-1 cell line (41). It is likely that the apparent two classes of lactoferrin receptors reflect at least in part the relative structural complexity of the 78-kDa lactoferrin molecule (24–26). Of note, the apparent number of lactoferrin receptor molecules expressed by eosinophils is less than that reported for other cells (34, 35, 37–39, 41). Although complete saturation was not achieved in the binding experiments using ^{125}I-labeled lactoferrin, the results of the flow cytometric analysis indicate that binding of the lactoferrin by eosinophils is saturated following incubation with 30 μg/ml (~0.4 μM) lactoferrin. Significantly, soluble lactoferrin does not activate the eosinophils as measured by superoxide production, and in concentrations up to 100 μg/ml (~1 μM) does not block eosinophil activation by immobilized lactoferrin. Together, these results suggest that lactoferrin-induced activation of eosinophils requires aggregation of a relatively low affinity lactoferrin receptor. It is of interest in this context that a lactoferrin receptor with a dissociation constant of ~200 nM has been described for neutrophils (34), but immobilized lactoferrin does not stimulate neutrophil superoxide production.

Lactoferrin is a member of the transferrin family of proteins and shares 60% sequence identity with serum transferrin (42), but immobilized transferrin did not stimulate eosinophil activation as measured by superoxide production. Transferrin also did not block the binding of ^{125}I-labeled lactoferrin by eosinophils. Lactoferrin contains a glycosaminoglycan binding site near its amino terminus (25, 26) that is absent in transferrin (42). This site has been implicated in a low affinity binding of lactoferrin by THP-1 cells (41) and also mediates binding of LPS by lactoferrin (11). The results presented here for heparin and chondroitin sulfate indicate that the glycosaminoglycan-binding site probably does not play a role in the eosinophil activation by immobilized lactoferrin. Heparin at a concentration of 1 mg/ml caused only modest inhibition (25%) of eosinophil superoxide production stimulated by 30 μg/ml immobilized lactoferrin. The inhibition, however, was not specific for immobilized lactoferrin, as heparin also caused similar inhibition of superoxide production stimulated by immobilized secretory IgA. The lower concentrations of heparin and none of the concentrations of chondroitin sulfate inhibited eosinophil superoxide production stimulated by immobilized lactoferrin. Lactoferrin and transferrin also differ slightly in the composition of their *N*-linked oligosaccharides, specifically in the presence of a fucose (α-1,6) residue in the core of the lactoferrin *N*-linked oligosaccharides (24). Participation of the fucosyl moieties, or indeed the *N*-linked carbohydrate moieties in general, in eosinophil activation by immobilized lactoferrin is excluded by the finding that deglycosylated lactoferrin also stimulates eosinophil superoxide production when immobilized onto a surface. Similar to the findings reported here, the high affinity binding of lactoferrin to the human promonocytic U937 cell line also occurs independently of fucosyl or glycosyl residues and was not blocked by heparinase treatment of the cells (37). Also similar to the findings here, transferrin does not inhibit the binding of lactoferrrin by HL-60 cells before or after induced differentiation toward monocyte/macrophage-like cells, by human monocytes, or by U937 cells (35–37).

Immobilized lactoferrin, although approximately one-third as potent as immobilized secretory IgA, is on occasion (Fig. 2*B*) nearly as efficacious as immobilized secretory IgA in stimulating eosinophil superoxide production and EDN release. Immobilized secretory IgA is one of the most potent stimuli for eosinophil superoxide production and degranulation (16). The increased potency of immobilized secretory IgA relative to either immobilized IgG or immobilized serum IgA (16) reflects the capacity of immobilized secretory component to also stimulate eosinophil superoxide production and degranulation (17). Interestingly, the responses stimulated by immobilized lactoferrin and immobilized secretory component share a trait in common. Immobilized lactoferrin and immobilized secretory component each stimulate eosinophil superoxide production, but not neutrophil superoxide production (Fig. 2*A*) (17). Eosinophil activation by immobilized secretory component is correlated with the presence of a putative 15-kDa receptor for secretory component on eosinophils that is absent in neutrophils (43). The possibility that immobilized lactoferrin may cross-react with the putative receptor for secretory component on eosinophils (43) cannot yet be excluded. It is of interest, however, that *S. pneumoniae* possess distinct and specific receptors for lactoferrin and secretory component (44–46), thus at least raising the possibility that lactoferrin and secretory component may likewise recognize distinct receptors on eosinophils.

Eosinophils are postulated to contribute to the pathogenesis in asthma and other allergic diseases through the release of their granule contents as well as production of reactive oxygen intermediates and lipid products, including leukotriene C_4 (3, 15). The capacity of immobilized lactoferrin to stimulate eosinophil superoxide production, degranulation, and leukotriene C_4 production suggests that lactoferrin adherent to the surface epithelium may constitute one mechanism for initiating these events within the airway. Moreover, the present results along with the activity of immobilized secretory IgA (16, 17) and the finding that Clara cell secretory 10-kDa protein can limit eosinophil-associated lung inflammation (47) indicate that prominent constituents within the airway surface liquid may contribute to the regulation of eosinophil activation within the airway. Although concomitant neutrophil infiltration and activation within the lungs could constitute an additional source of lactoferrin for eosinophil activation, it is worth noting that oxidizing pollutants have been reported to increase lactoferrin synthesis by bronchial epithelial glands (48). Further, the finding that eosinophil cationic protein stimulates lactoferrin release by serous glands in explants of human nasal mucosa (49) raises the possibility that eosinophil activation by immobilized lactoferrin may provide feedback reinforcement for additional or persistent eosinophil activation within the airway.

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