Feedback Regulation of Granulopoiesis: Polymerization of Lactoferrin Abrogates Its Ability to Inhibit CSA Production

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Neutrophil extracts were prepared from the peripheral blood of 40 normal volunteers and tested for their ability to inhibit CSA production by mononuclear leukocytes. Highly dilute neutrophil extracts inhibited CSA production/release, while extracts selectively depleted of lactoferrin by antibody affinity chromatography did not. In addition, higher concentrations of neutrophil extracts and higher doses of lactoferrin (10^{-5} to 10^{-4} M) failed to inhibit CSA production/release. We found no evidence of CSA or CSA-enhancing factors in either our lactoferrin or our neutrophil extracts. However, using gel chromatography and rate zonal density sedimentation, we noted that lactoferrin undergoes concentration-dependent polymerization at 10^{-9} to 10^{-6} M in tissue culture medium and that while monomeric lactoferrin effectively inhibits CSA production/release in vitro, the polymeric form does not. Thus, while we have confirmed that lactoferrin is the activity in neutrophil extracts that inhibits CSA production, we have also found that lactoferrin undergoes reversible polymerization at physiologic concentrations and that the polymerized molecule is inactive. The tendency of lactoferrin to polymerize in tissue culture medium and in vivo should be taken into account in any studies on its potential role as a physiologically relevant regulator of granulopoiesis.

**MATERIALS AND METHODS**

**Neutrophil Extracts**

Neutrophil extracts were prepared according to a modification of the technique described by Broxmeyer et al. High density cells were prepared by placing peripheral blood obtained from 40 consenting adult normal volunteers upon Ficoll-Paque (Pharmacia Fine Chemicals, Division of Pharmacia, Inc., Piscataway, N.J.). The red cells and neutrophils were separated by dextran sedimentation and concentrated to 16 x 10^6/ml. The cells were freeze-thaw lysed, and the extract was centrifuged at 160,000 g for 18-19 hr. The extracts were diluted (1:10 to 1:100,000) with buffered saline and frozen at -80°C.

**Lactoferrin**

Highly purified human breast milk lactoferrin and rabbit anti-human lactoferrin antibodies were prepared as previously described. Lactoferrin with an iron saturation of 85% was used in the studies below. The preparation was free of endotoxin.

**Lactoferrin Depletion**

Rabbit anti-human lactoferrin antibody affinity columns were prepared as previously described, and neutrophil extracts (from 20 adult volunteers) were depleted of lactoferrin using these columns. All fetal calf serum used in the colony growth assays below had been depleted of lactoferrin using these columns as previously described.

**Colony-Inhibiting Activity Assays**

Multiple doses of neutrophil extracts, lactoferrin-depleted neutrophil extracts, and saturated lactoferrin (14 doses from 10^{-3} M to 10^{-9} M) were used in each of the colony (CFU-GM) inhibiting activity assays, which we have described previously, to measure the degree to which lactoferrin inhibited colony-stimulating activity production. The first assay was an autostimulatory assay wherein single layer agar (0.3% in McCoy's 5A medium with 15% FCS) plates of low density (Ficoll-Paque) bone marrow cells were cultured for 7 days at 37.5°C in 7.5% CO2 in air. Colonies and...
clusters were counted in quadruplicate plates. Colony growth in this system is completely dependent on the production of CSA by monocytes and T lymphocytes in the plated marrow sample. Control studies were performed in which low density marrow cells were cultured with 10% leukocyte conditioned medium prepared according to a method described by Iscove.

The second assay consisted of a double layer agar culture with peripheral blood mononuclear leukocytes (5-10 x 10^7/ml, 0.5% agar in McCoy's 5A and 15% FCS) as feeder layers over which autologous T-lymphocyte-depleted low density bone marrow cells (0.3-1.0 x 10^7/ml) were plated in 0.3% agar medium. Lactoferrin or neutrophil extracts were added to the feeder layers. Control plates had acellular underlayers overlaid with the same target cells.

In the third assay the effects of neutrophil extracts or lactoferrin (10^-6 - 10^-4 M) on CSA production by peripheral blood mononuclear cells (0.5-1.0 x 10^7/ml) in RPMI 1640 with 15% FCS (x 7 days) were analyzed. The leukocyte conditioned media were harvested and added to single layer methylcellulose or agar cultures of autologous T-lymphocyte-depleted low density bone marrow cells as previously described. Marrow and blood samples were obtained from normal volunteers who had given signed informed consent, and the cells were processed as previously described. Inhibition of colony growth was considered significant in any experiment if the test material effected a 20% or more inhibition and p < 0.01 by the Student's t test.

Chromatography

Two milliliters of 10^-3 M lactoferrin in serum-free medium was layered over Sephacryl S-300 (Pharmacia Fine Chemicals, Piscataway, N.J.). The eluates were monitored at 280 nm. Eluates were tested for lactoferrin by radioimmunoassay and for colony-inhibiting activity in the autostimulatory colony growth assay (see above). The column was calibrated with ferritin, aldolase, and ovalbumin.

In two experiments, the lactoferrin was chromatographed over Sephacryl S-300 equilibrated with phosphate-buffered saline supplemented with 10 mM sodium EDTA.

Rate Zonal Density Sedimentation

In two separate experiments, various doses of cold lactoferrin (10^-11 - 10^-3 M) were mixed with 10^-14 M 125I-labeled lactoferrin in RPMI 1640 and was layered on a continuous 10%–40% sucrose density gradient. Centrifugation was carried out at 4°C, and 105,000 g for 18 hr. Each experiment consisted of 5 or 6 tubes containing lactoferrin and one tube containing molecular weight markers. In these, as in other studies, the monomer sedimented in a position consonant with a molecular weight of 78,000, while the polymer was approximately 310,000. These experiments were designed subsequent to the gel chromatography experiments to measure the concentrations of lactoferrin at which polymerization occurs.

RESULTS

Thirty-eight of 40 neutrophil extracts inhibited clonal (CFU-GM) growth when the extract was added to cultures of CSA-producing cells. Specifically, when autologous marrow cells or peripheral blood leukocytes were cultured in the presence of a 1:10,000 dilution of neutrophil extract, CSA production, as assessed using conditioned media, feeder layer assays or autostimulatory assays, was inhibited in 38 of 40 experiments. In no case did neutrophil extracts inhibit colony growth stimulated by preformed CSA.

Serial dilutions of neutrophil extracts were tested in the feeder layer assay and we noted that significant inhibition of clonal growth occurred only with 1:100,000 and 1:10,000 dilutions. More concentrated extracts failed to inhibit colony growth (Fig. 1). Moreover, high dose extracts and high dose lactoferrin-depleted extracts failed to stimulate colony growth in the absence of feeder layers. Neutrophil extract samples depleted of lactoferrin on an antibody affinity column failed to inhibit colony growth, but nondepleted samples did (Fig. 2).

Because we had confirmed in this study Broxmeyer's observation that neutrophil-derived colony inhibitory activity is lactoferrin and had also noted loss of activity with high doses of neutrophil extracts, we tested the hypothesis that high dose lactoferrin would lose its inhibiting activity. As shown in Fig. 3, human breast milk lactoferrin inhibits feeder layer provision of CSA by 40% in 10 separate experiments, but the effective doses range from 10^-17 M to 10^-10 M. Higher lactoferrin titers are not inhibitory.

Absorbancy curves of sequential volumes of effluent from a Sephacryl S-300 gel on which 10^-3 M lactoferrin had been layered showed two peaks (Fig. 4), one corresponding to a molecular weight of 76,000 and the other to 300,000. In the presence of EDTA, only the monomeric peak is noted (Fig. 4). When the polymerized lactoferrin obtained from the column was used in colony inhibition assay (autostimulatory), it failed to inhibit colony growth unless it was diluted to 10^-16 M or greater (at which point the polymer had dissociated into a monomer).

The results of density gradient centrifugation are
Depleted ferrin is the neutrophil-derived colony-inhibiting activity (Fig. 2), and in a recently reported study have documented that lactoferrin specifically prevents the recruitment by mononuclear phagocytes of T lymphocytes to produce CSA. We have consistently noted that high doses of neutrophil extracts and high doses of but does not affect inhibition of colony growth by lactoferrin fail to restrain CSA production, whereas directly inhibiting CFU-GM or altering the activity of lower doses do (Fig. 1 and 3). Because Broxmeyer's group has reported that their lactoferrin preparation exerts inhibitory activity to $10^{-6}$ M and that in their laboratory our preparation loses activity at $10^{-9}$ M, we suggest that lactoferrin exists as a tetramer. The dotted line represents the elution pattern of lactoferrin in the presence of EDTA.

DISCUSSION

Results of this study confirm many of Broxmeyer's original observations. For example, we have confirmed that an activity in neutrophil extracts inhibits CSA production or release by mononuclear leukocytes but does not affect inhibition of colony growth by directly inhibiting CFU-GM or altering the activity of the CSA molecule. We have confirmed that lactoferrin is the neutrophil-derived colony-inhibiting activity (Fig. 2), and in a recently reported study have documented that lactoferrin specifically prevents the recruitment by mononuclear phagocytes of T lymphocytes to produce CSA. We have consistently noted that high doses of neutrophil extracts and high doses of lactoferrin fail to restrain CSA production, whereas lower doses do (Fig. 1 and 3). Because Broxmeyer's group has reported that their lactoferrin preparation exerts inhibitory activity to $10^{-6}$ M and that in their laboratory our preparation loses activity at $10^{-9}$ M and above (H.E. Broxmeyer, personal communication), we...
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initially feared that the apparent differences in our preparations might have reflected contamination of neutrophil extracts and lactoferrin with CSA or factors capable of stimulating CSA production (factors that might mask lactoferrin’s inhibitory activity). Based on further studies, we do not believe that these explanations are likely because (1) high dose neutrophil extracts, high dose lactoferrin, and high dose lactoferrin-depleted neutrophil extracts fail to stimulate colony growth of macrophage-depleted, T-lymphocyte-depleted low density bone marrow cells; (2) results of studies using permeation chromatography, polyacrylamide disc electrophoresis, and immunoelectrophoresis all suggest protein homogeneity;\(^1\) (3) no endotoxin is detectable in our lactoferrin preparations;\(^3\) and (4) neutrophil extracts and lactoferrin fail to stimulate CSA production by mononuclear phagocytes. For these reasons the studies reported herein were designed, in part, to test the hypothesis that a primary alteration of lactoferrin structure might account for the observed alteration in activity. Using gel chromatographic techniques, we have found that in the presence of divalent cations (which in other studies have proved to be specifically calcium), lactoferrin undergoes polymerization (Fig. 4) and that the polymeric form fails to inhibit CSA production/release unless it is diluted to the point of dissociation. Using sucrose rate zonal density sedimentation, the polymerization process appears to occur at concentrations in excess of \(10^{-11} \text{ M}\) and the molecule seems to be in a completely polymerized form at \(10^{-9} \text{ M}\) (Fig. 5), at which point lactoferrin loses its capacity to restrain CSA production (Fig. 3). The mechanisms whereby polymerized lactoferrin fails to exhibit activity are unknown. Future studies on the tertiary and quaternary molecular changes of lactoferrin will be of value, as they might lead to the identification of active sites on the molecule and the understanding of the role iron plays in molecular activity.

The relevance of the polymerization phenomenon to the regulation of steady-state granulopoiesis is unknown, but polymerization has been described in vivo\(^1,12,16,17\) and the polymeric form predominates in fluids from inflammed organs in which lactoferrin levels are characteristically high.\(^11,12,16-18\) One might propose teleologically that when inflammation is present, neutrophil-derived lactoferrin should not be able to restrain granulocyte production, but previously reported in vitro studies indicate that endotoxin or antigen-stimulated cells not only increase baseline CSA production, but become refractory to the inhibitory influences of lactoferrin.\(^5\) Either or both of these mechanisms may operate in vivo to override lactoferrin’s inhibitory activity in states of inflammation. It is widely presumed, and in vitro evidence has been reported which suggests, that feedback regulation of granulopoiesis is defective in patients with chronic granulocyte leukemia.\(^6\) Our results suggest that most if not all of the lactoferrin in the serum of patients with this disease would be polymeric as the serum levels are characteristically high (about \(3 \times 10^{-8} \text{ M}\)),\(^10\) at which point the protein should exist primarily as a polymer incapable of inhibiting CSA production. The role that polymerization might play in the pathophysiology of this disorder is not known. Indeed, it should be recognized that we have no evidence to date that the polymer-to-monomer transition plays any role in regulation of granulopoiesis. In fact, the in vivo role of lactoferrin itself is by no means proved.

There has been some skepticism expressed even on the issue of lactoferrin’s in vitro activity\(^9,20\) by investigators who have regarded the threshold dose of \(10^{-17} \text{ M}\) originally described by Broxmeyer\(^4\) as tentative (fetal calf serum may contain more than \(10^{-17} \text{ M}\) and by others who have failed to demonstrate lactoferrin-mediated suppression of CSA production.\(^20\) In this and another related study\(^8\) using a lactoferrin-free culture system, we have confirmed the reported threshold dose and have confirmed that neutrophil colony-inhibiting activity is lactoferrin. In addition, we have reported two new features of lactoferrin’s activity that may explain at least some negative results in other laboratories. First, T lymphocytes and mononuclear phagocytes are required for lactoferrin to express its inhibitory activity,\(^6\) and second, lactoferrin’s activity is lost when the molecule polymerizes at high doses. Although the study reported here does not indicate that the phenomenon of molecular self-aggregation or indeed molecular lactoferrin itself have relevance to the regulation of granulopoiesis in vivo, our results do indicate that the tendency of lactoferrin to polymerize both in vivo and in tissue culture medium should be taken into account in any studies on the potential role of this molecule as a regulator of steady-state granulopoiesis.

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