IDENTIFICATION OF LACTOFERRIN AS THE GRANULOCYTE-DERIVED INHIBITOR OF COLONY-STIMULATING ACTIVITY PRODUCTION*

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A number of factors have been implicated in positive and negative feedback mechanisms regulating the production of granulocytes and macrophages in vitro and in vivo (1). A family of glycoproteins collectively termed colony-stimulating activity (CSA)[1] stimulates colony formation in vitro (1). We have recently documented the existence of a negative feedback control mechanism mediated by a glycoprotein inhibitory factor derived from mature granulocytes which limits colony formation in vitro and granulopoiesis in vivo by decreasing the production and release of CSA from normal and leukemic monocytes and macrophages (2–6). From our recent interest in the postulated interaction of iron and iron-binding proteins with cells of the immune system (7, 8), we have noted that this colony inhibitory factor (CIF) is similar in many respects to lactoferrin (LF), the iron-binding protein first isolated from milk and also present in epithelial secretions and mature granulocytes (9–14). These include: (*a*) a similar biochemistry: CIF and LF are glycoproteins with mol wt ≅80–100,000 (6, 15) and isoelectric focus points of ≅ pH 6.0–6.5 (6, 13, 16); (*b*) a similar site of production: both are found in mature granulocytes and are quantitatively deficient in the mature granulocytes of patients with acute and chronic leukemia and myelodysplasia (2, 3, 17–20); and (*c*) a similar site of action: both interact with monocytes and macrophages (2, 21, 22). In the present paper we identify CIF as LF and present evidence that strongly implicates LF as a physiological regulator of granulopoiesis.

Materials and Methods

Physical Separation of Cells into Functional and Morphologic Populations. Bone marrow and blood cells from healthy volunteers were separated on the basis of their density and/or adherence to plastic surfaces to obtain populations of granulocyte and macrophage colony-forming cells

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[1] *Abbreviations used in this paper:* ApoLF, LF depleted of iron; BFU-e, erythroid burst-forming cell; CFU-c, granulocyte and macrophage colony-forming cell; CFU-e, erythroid colony-forming cell; CIF, inhibitor of CSA production; CSA, granulocyte and macrophage colony-stimulating activity; FCS, fetal calf serum; LF, lactoferrin; LIA, leukemia-associated inhibitory activity; LPS, bacterial lipopolysaccharide; native LF, LF saturated with ≅8% iron; NIA, neutropenia-associated inhibitory activity; PBS, phosphate-buffered salt solution; TF, transferrin.

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(CFU-c) devoid of CSA-producing cells and mature granulocytes, CSA-producing cells depleted of mature granulocytes and mature granulocytes alone (2, 17). Cells were separated by a density cut in bovine serum albumin (density, 1.070 g/cm^3; osmolarity, 270 mosmol). CFU-c were present in the nonadherent low density fraction (<1.070 g/cm^3) and CSA-producing cells were found in the adherent low density fraction. Mature granulocytes (90–100% pure) were present in the high density fraction (>1.070 g/cm^3).

Assay Procedures

A. CSA PRODUCTION. Human active CSA was obtained from endogenous marrow CSA-producing cells, peripheral blood monocytes, and placental cells, all of human origin (2, 17, 23). Human adherent low density blood cells which contained >90% monocytes as judged by morphological criteria and histochemical affinity for α naphtyl acetate esterase and neutral red were suspended at 2–5 × 10^5 cells/ml and allowed to condition McCoys medium (Memorial Sloan-Kettering Institute, N. Y.) containing 10% fetal calf serum (FCS) (Microbiological Associates, Walkersville, Md.) with CSA for 2–4 days.

Murine active CSA was obtained from medium conditioned by WEHI-3 cells, a murine myelomonocytic leukemia cell line (4, 24), and from heart and lung of mice treated with cyclophosphamide. WEHI-3 cells were suspended at 1–2 × 10^5 cells/ml and allowed to condition medium as above. The entire heart was removed from each treated mouse, rinsed in McCoy's medium, sliced into six to eight large parts, and allowed to condition 5 ml of medium for 3–4 days. The intact lung from each treated mouse was also allowed to condition 5 ml of medium with CSA.

Dilutions of test material were placed in the dishes at time zero with the human monocytes or WEHI-3 cells or 1 and 2 days after the cells had started to condition the culture medium.

Human and murine cell conditioned medium were assayed for CSA as in C either undiluted or diluted at 0.1 ml before or after dialysis in spectropor membrane tubing (mol wt cutoff ≅6–8,000 daltons; Spectrum Medical Industries Inc., Los Angeles) against 10 mM sodium phosphate buffer pH 7.0 (2 × 100 vol). Conditioned medium was passed through a Millipore filter (Millipore Corp., Bedford, Mass.) and stored at −20°C until assay.

B. COLONY. (>50 cells/clone) and cluster (3–50 cells/clone) formation of bone marrow CFU-c stimulated by endogenous CSA-producing cells (spontaneous assay). Human bone marrow cells depleted of mature granulocytes (density <1.070 g/cm^3) and bone marrow cells of mice recovering from cyclophosphamide at a time when mature granulocytes were absent were suspended at 3 × 10^5 cells in 1 ml of a 0.3% agar culture medium (Difco Laboratories, Detroit, Mich.) with enriched McCoy's medium containing 10% heat-inactivated FCS. Inhibition of human colony formation was assessed by addition of 0.1 ml of various dilutions of test material.

C. COLONY AND CLUSTER FORMATION OF BONE MARROW CFU-C STIMULATED BY EXOGENOUSLY SUPPLIED CSA. In the human assay, 1–2 × 10^5 nonadherent low density bone marrow cells (depleted of endogenous CSA-producing cells) per milliliter in 0.3% agar culture medium were stimulated by CSA derived from human blood leukocytes in a 0.5% agar feeder layer (prepared 3–7 days before addition of target cells and test material), or a constant amount of stimulatory activity derived from human blood monocyte or human placental cell-conditioned medium. In the mouse assay, 0.5–1.0 × 10^5 unseparated or nonadherent mouse bone marrow cells were stimulated by murine cell conditioned medium. Dilutions of inhibitory test material were made and 0.1 ml added to the culture plates.

D. IN VIVO INHIBITORY ASSAY. 5–7-wk old conventionally outbred female Swiss mice (CD-1, Charles River Breeding Laboratories, Inc., Wilmington, Mass.) and 3–6 mo-old C57Bl/6 mice (The Jackson Laboratory, Bar Harbor, Maine) were treated with 200 mg cyclophosphamide/kg body wt and served as an in vivo model for inhibition of rebound granulopoiesis as described in reference 5.

E. ERYTHROID COLONY ASSAYS. Erythroid colony assays were carried out according to the method of Iscove et al. (Friedrich Miescher-Institut, Basel, Switzerland) (25, 26). Human unseparated or nonadherent low density bone marrow cells were plated at 1 or 2 × 10^5/plate and murine bone marrow cells were plated at 2 × 10^5/plate. Cells were plated in alpha medium containing a final concentration of 0.8% methylcellulose, 30% FCS, 1% bovine serum albumin, and 1 U/ml of human urinary erythropoietin. The erythropoietin was kindly supplied

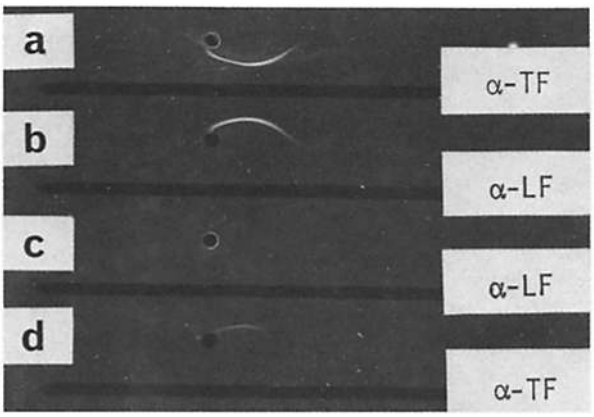

FIG. 1. Immunoelectrophoresis of normal human serum, lactoferrin, and transferrin developed against anti-transferrin and anti-lactoferrin antisera. Well a, normal human serum; well b, purified human transferrin; well c, normal human serum; well d, purified human lactoferrin (photograph of unstained preparation).

by Dr. Norman Iscove. Individual colonies were plucked, placed on glass slides, stained with benzidine, and counter stained with Wright-Giemsa to confirm the erythroid nature of the colonies.

Culture Conditions and Scoring of In Vitro Cultures. In vitro cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Myeloid colonies (>50 cells) and clusters (3–50 cells) were scored at days 7–12 for human cells and days 5–7 for mouse cells. Human erythroid colony-forming units (CFU-e) and burst-forming units (BFU-e) were scored at 7 and 14 days, respectively. Murine CFU-e and BFU-e were scored at 2 and 9 days, respectively.

Statistics. The probability of differences between samples was determined with the use of the Student's *t* test. Three to five plates were scored for the CFU-c assay and four to eight plates were scored for the CFU-e and BFU-e assays. In the assay for CSA production, the supernate from three to five plates was each assessed separately with three to five agar culture plates scored for each conditioned medium plate. The cells from the tissues and organs of each mouse were set up separately using five plates per point. The means ± 1 SEM of each experimental point were obtained from the average values of five mice.

Preparation of Cell-Free Granulocyte Colony Inhibitory Factor (CIF). Cell extracts were prepared by suspending mature granulocytes at concentrations of 1.0–20.0 × 10^6 cells/ml in serum-free enriched McCoy's 5A medium and lysing the cells by rapid freezing and thawing (one to three times to 0°C). Extracts were passed through a Millipore filter (pore size, 0.45 μM). CIF was also obtained when mature granulocytes were allowed to condition serum-free medium for 1–2 days. The extracts or conditioned media were frozen and used as needed or subjected to purification by ultracentrifugation, DEAE-Sephadex chromatography, sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, and isoelectric focusing as previously described (6).

Agents Used

ANTISERA. The purified immunoglobulin fractions of rabbit anti-human LF and transferrin (TF) were obtained from Dako Immunoglobulins (Accurate Chemical & Scientific Corp., Hicksville, N. Y.) and goat anti-human LF antiserum (27) was generously supplied by Dr. Jiri Mesteky (University of Alabama, Birmingham, Ala.). Anti-LF immunoglobulin gave a single precipitation line after immunoelectrophoresis (Fig. 1) or Ouchterlony double diffusion (not shown) against purified LF and human skim milk but did not cross-react with purified TF or normal human serum. Conversely, anti-TF immunoglobulin gave a single sharp precipitation line against purified TF or normal human serum but did not cross-react with purified LF. Mature granulocyte extracts cross-reacted with anti-LF but not anti-TF (data not shown).

Rabbit anti-human Ia antisera prepared as described in (28) was generously supplied by Dr. Robert Winchester of Rockefeller University, N. Y.

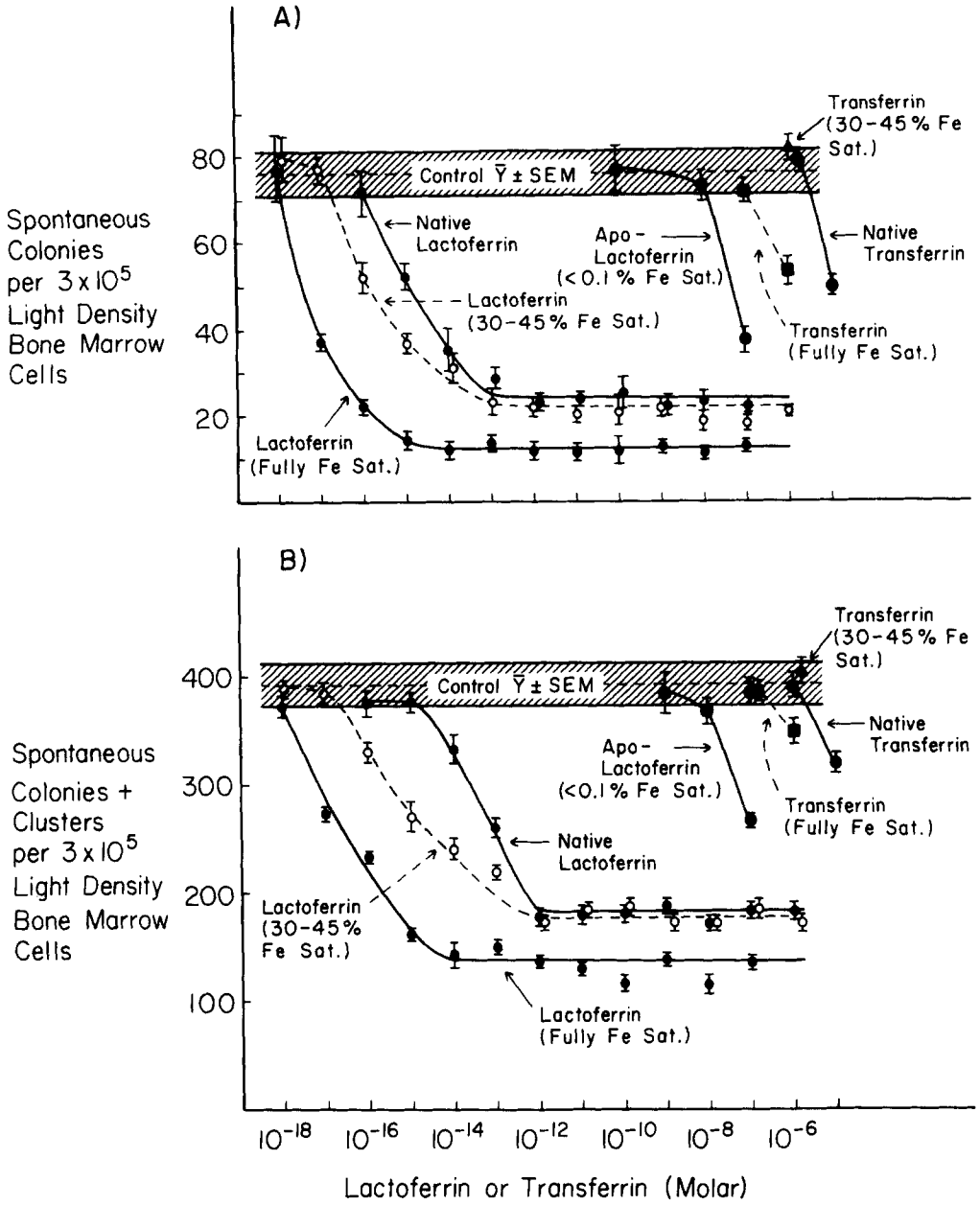

FIG. 2. The inhibitory activity of lactoferrin enhanced by increased iron saturation. Native lactoferrin is 8% iron saturated. Brackets represent ± 1 SEM. 10–15% Fe saturation curve for lactoferrin was superimposable with that of native lactoferrin and is not shown. Sat, saturation.

Rabbit anti-human β_2-microglobulin was purchased from Dako Immunoglobulins. Rabbit anti-human IgA was purchased from Behring Diagnostics (Somerville, N. J.), and rabbit anti-human complement (C_3) and rabbit anti-human α_2-macroglobulin were purchased from N. L. Cappel Laboratories Inc., (Cochranville, Pa.). The antisera were extensively dialyzed at 4°C against phosphate-buffered salt solution (PBS) to remove previously added sodium azide before use in the in vitro tests.

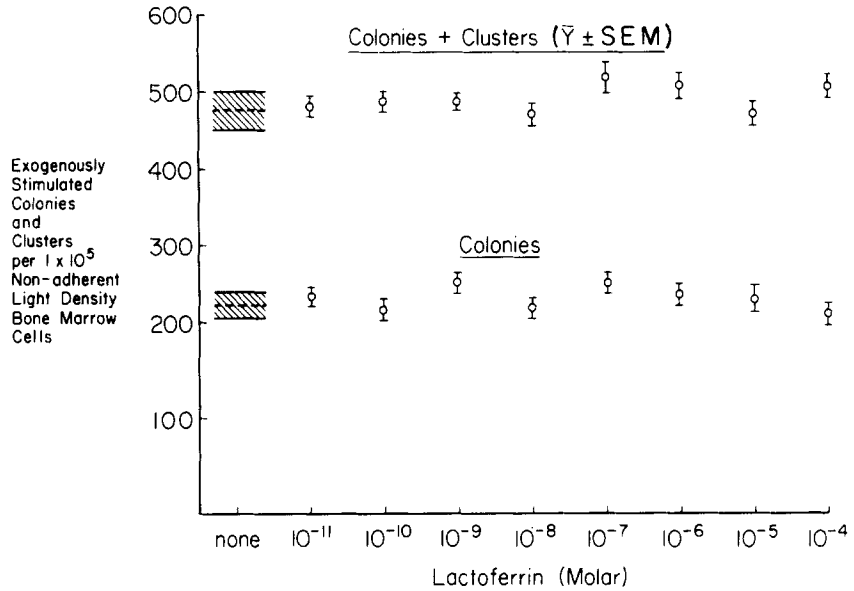

FIG. 3. Lack of an inhibitory effect of lactoferrin on exogenously stimulated colony and cluster formation of bone marrow cells depleted of the adherent endogenous CSA-producing cells. Blood leukocyte feeder layers were 5 days old before plating the target marrow plus lactoferrin.

Commercial LF, prepared by ammonium sulphate precipitation of human skim milk (29) was obtained from Calbiochem (San Diego, Calif.). LF was purified in this laboratory from human skim milk by the method of Johannson (30) as adapted by Queringean, Masson, and Heremans (15). Purified human TF was purchased from Behring Diagnostics.

Bacterial Lipopolysaccharide. (*Salmonella typhosa* W0901) (LPS) was purchased from Difco Laboratories (Detroit, Mich.).

Iron Removal and Saturation of LF. Apo-LF was prepared by dialysis at 4°C against 100 vol of 0.1 M citric acid (31, 32). After 48 h, the protein was dialyzed with two changes against PBS at 4°C.

Iron-Saturated LF and TF. Iron-saturated LF and TF were prepared in two ways. In the first method, native LF was dialyzed for 48 h at 4°C, first against 500 vol of saturated ferrous ammonium sulphate and then against PBS at 4°C (32). In the second method, 10 μl of various dilutions of a 10-mM ferric citrate solution were added to three 1-ml samples of LF or TF at 100 μg protein/ml to yield either fully saturated, partially saturated (≅30–45% saturated), or poorly saturated (≅8% saturated) solutions. The fully saturated LF from the first and second methods were equally potent when assayed against CSA production.

Isoelectric Focusing. Preformed polyacrylamide gel plates (245 × 110 × 1 mm) containing 2.4% wt/vol ampholytes (pH range of 3.5–9.5) were purchased from LKB (Sweden) (33). The test materials were applied on the gel surface and the gel was focused for ≅2 h after which 1-cm squares were cut along the gel edge from the cathode to the anode. These were each placed in 1 ml of distilled water overnight and used to measure the pH. The remainder of the gel was cut into 1-cm strips each of which was further cut into 11 1-cm squares. Each square was eluted overnight in 1 ml of PBS and the eluate dialyzed extensively against PBS.

Results

Comparative Analysis of the In Vitro Action of CIF and LF on Colony and Cluster Formation. Conditioned media and extracts from human and murine mature granulocytes inhibit spontaneous colony and cluster formation of human and murine bone marrow cells by suppressing endogenous CSA production (2). In preliminary experiments it

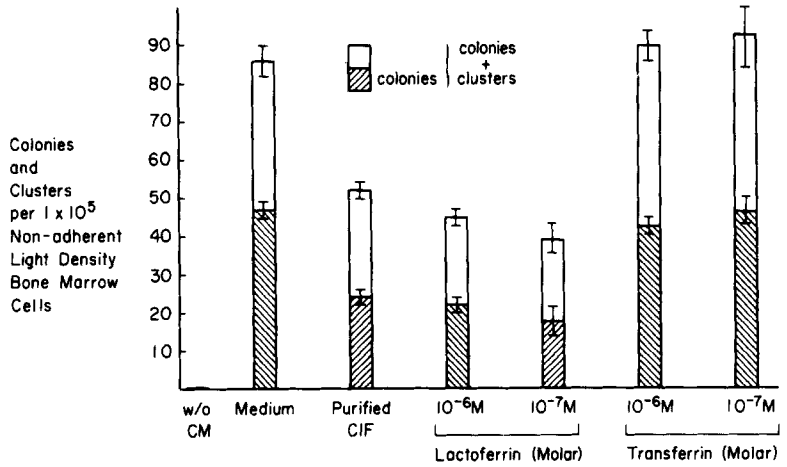

FIG. 4. Medium conditioned by adherent mononuclear blood cells in presence of CIF, lactoferrin, or transferrin before addition to target cells. Inhibitory effect of lactoferrin and CIF on the elaboration of CSA from adherent mononuclear blood cells (90% monocytes). Transferrin had no effect on CSA production.

was noted that native LF (8% iron saturated) also reduced spontaneous colony and cluster formation of human bone marrow cells. One of four similar dose-response assays is presented in Fig. 2. Native LF maximally inhibited 71% of colonies (Fig. 2a) and 55% of total aggregates (Fig. 2b). The inhibitory activity fell off but was still detectable between 10^{-13} and 10^{-14} M for colonies, 10^{-12} and 10^{-13} M for colonies and clusters, and all activity was lost by 10^{-14}–10^{-16} M LF.

Effect of Iron Saturation on the Action of LF. It was readily apparent from Fig. 2 and four other experiments that the percent iron saturation of LF markedly influenced inhibitory activity. LF depleted of iron (Apo-LF) was only inhibitory at concentrations $> 10^{-7}$ M, but fully iron-saturated LF extended the dilution of LF that inhibited spontaneous colony and cluster formation (to 10^{-17} M) and also increased the plateau levels of inhibition to 84% and 65% for colonies and total aggregates, respectively. As shown in Fig. 2 (and in three additional experiments not shown), human TF had minimal inhibitory activity (10–34%) at concentrations $> 10^{-6}$ M. Fully saturating TF with iron did not enhance this minimal inhibitory activity.

Site and Mode of Action of LF. To determine the mechanism of LF action, two other assay systems were employed. In the first, endogenous CSA-producing cells were removed from low density marrow cells by adherence to plastic and the effect of LF was monitored on colony and cluster formation of nonadherent marrow cells stimulated by exogenous CSA. In the second assay, the effect of LF on CSA production was assessed.

The results of one of four similar experiments using blood leukocyte feeders as a source of exogenous CSA is shown in Fig. 3. LF (apo to fully iron saturated) at concentrations between 10^{-4} and 10^{-11} M had no effect on exogenously stimulated colony and cluster formation of bone marrow cells depleted of adherent endogenous CSA-producing cells. TF at these concentrations also had no influence on the assay (data not shown). In six other experiments, 10^{-4}–10^{-8} M LF had no effect on colony and cluster formation of nonadherent bone marrow cells stimulated by various

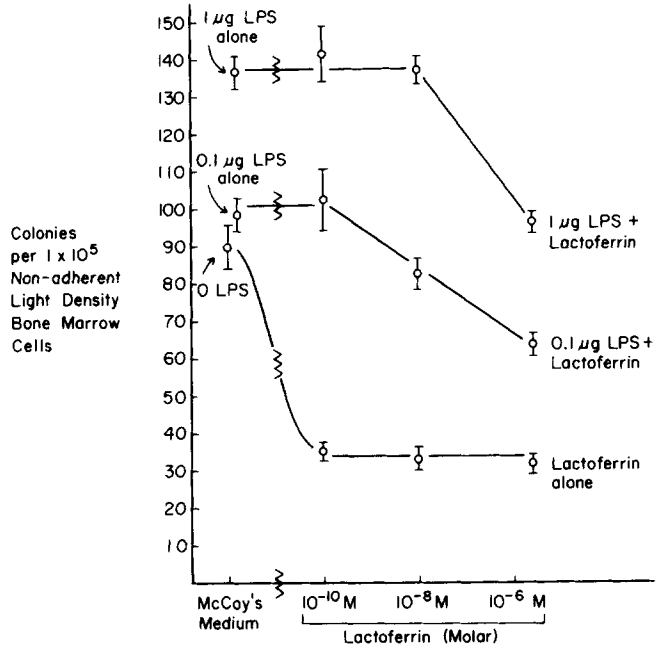

FIG. 5. Medium conditioned by adherent mononuclear blood cells in presence of lactoferrin and/or bacterial LPS before addition to target cells. LPS abrogation of the inhibitory effect of lactoferrin on CSA production.

dilutions of human placenta-conditioned medium CSA (in the range of 3–150 colonies and 10–450 total aggregates). This suggested that LF was not acting to inactivate performed CSA or to decrease the sensitivity of CFU-c to CSA.

The capacity of LF to inhibit CSA production was tested by incubating LF with human adherent mononuclear blood cells. As shown in Fig. 4, purified CIF and LF at 10^{-6} and 10^{-7} M suppressed CSA production, but TF at 10^{-6} and 10^{-7} M was without effect. Preincubation of mononuclear blood cells with 10^{-7} M LF for ½ h before washing reduced CSA production to the same extent as leaving LF in the medium through the 3-day conditioning medium period. Both procedures resulted in 60% inhibition of CSA production. The suppressive effect of LF was reversible and could be blocked and/or overcome in a concentration-dependent fashion by bacterial LPS activation of human adherent mononuclear blood cells. As demonstrated in Fig. 5, more LPS was needed to overcome the effect of increasing concentrations of LF and vice versa. 0.1 μg LPS decreased the inhibitory influence of LF but did not increase CSA production, whereas 1 μg of LPS both stimulated CSA production and reversed the inhibition. In another experiment, shown in Table I, in which human adherent mononuclear blood cells and murine WEHI-3 cells were exposed to 10^{-8} M LF at day 0 and 1 μg LPS at either day 0, 1, or 2, LPS reversed the inhibitory activity of LF. The reversal was total if LPS was added at day 0, partial if LPS was added 1 day after LF, and nil if added at 2 days.

Identity of LF and CIF Shown by Isoelectric Focusing. Laboratory purified LF cross-reacted with anti-LF, but not anti-TF, in the Ouchterlony double-diffusion technique and suppressed CSA production by human adherent mononuclear blood cells (data

TABLE I

Abrogation of Inhibitory Action of Lactoferrin by Bacterial LPS

	CFU-c*	%Δ
Medium conditioned by human adherent mononuclear cells‡		
Control medium	76 ± 6	
Lactoferrin, 10^{-8} M (day 0)	21 ± 2	−72§
LPS, 1 μg (day 0)	111 ± 5	+46§
Lactoferrin, 10^{-8} M (day 0) + LPS, 1 μg (day 0)	120 ± 5	+58§
Lactoferrin, 10^{-8} M (day 0) + LPS, 1 μg (day 1)	48 ± 1	−37§
Lactoferrin, 10^{-8} M (day 0) + LPS, 1 μg (day 2)	20 ± 3	−74§
Medium conditioned by WEHI-3 cells‡		
Control medium	111 ± 4	
Lactoferrin, 10^{-8} M (day 0)	20 ± 2	−82§
LPS, 1 μg (day 0)	160 ± 5	+44§
Lactoferrin, 10^{-8} M (day 0) + LPS, 1 μg (day 0)	141 ± 6	+27§
Lactoferrin, 10^{-8} M (day 0) + LPS, 1 μg (day 1)	70 ± 3	−37§

* Colonies per 1×10^5 human nonadherent bone marrow cells stimulated by human cell conditioned medium and 1×10^5 C57Bl/6 mouse bone marrow cells stimulated by WEHI-3 cell-conditioned medium.

‡ Human conditioned medium collected after 3 days and WEHI-3 conditioned medium collected after 2 days.

§ Significant change $P < 0.05$.

TABLE II

Inactivation of CIF with Anti-Lactoferrin, but not with Anti-Transferrin

Experiment	Antiserum*	Dilution	Test material	Colonies + clusters‡	%Δ
1	None		McCoy's Medium	201 ± 11	−
	None		CIF-Crude§	104 ± 4	−48‖
	None		CIF-Purified§	130 ± 11	−35‖
	Anti-lactoferrin	10^{-2}	CIF-Crude	215 ± 6	+7
	Anti-lactoferrin	10^{-2}	CIF-Purified	203 ± 7	−1
2	None		McCoy's Medium	557 ± 12	−
	None		CIF-Crude	373 ± 5	−33‖
	None		CIF-Purified	357 ± 4	−36‖
	Anti-lactoferrin	10^{-1}	CIF-Crude	554 ± 10	−1
	Anti-lactoferrin	10^{-1}	CIF-Purified	518 ± 20	−7
	Anti-transferrin	10^{-1}	CIF-Crude	355 ± 10	−36‖
	Anti-transferrin	10^{-1}	CIF-Purified	377 ± 28	−32‖

* One part antiserum plus three parts test material were incubated at room temperature for 2–3 h before placing into test assay. The antiserum itself had no effect on the assay.

‡ Colonies and clusters per 3×10^5 light density human bone marrow cells stimulated by endogenous CSA (spontaneous assay) were scored after 10 days of incubation. Results were similar for colonies.

§ Crude CIF is initial mature granulocyte extract and purified CIF is extract subjected to DEAE Sephadex chromatography, SDS polyacrylamide gel electrophoresis, and isoelectric focusing as in reference 6.

‖ Significant change, $P < 0.05$.

not shown). This fraction, commercial LF, and purified CIF were subjected to isoelectric focusing and the capacities of these three materials to suppress CSA production were compared. Maximum inhibitory activity for the three preparations were found in the same area which corresponded to a pH range of 6.5 (data not shown), a pH value consistent with that determined by others for LF (14, 16).

TABLE III

Specificity of the Anti-Lactoferrin Inactivation of CIF and Lactoferrin*

Antiserum‡	Test material	Colonies and clusters§	%Δ
None	McCoy's medium	258 ± 9	–
None	CIF-Crude‖	130 ± 3	−50¶
None	CIF-Purified‖	112 ± 10	−57¶
None	Lactoferrin, 1 μg/ml	129 ± 10	−50¶
Anti-lactoferrin (unabsorbed)	CIF-Crude	252 ± 19	−2
Anti-lactoferrin (unabsorbed)	CIF-Purified	259 ± 4	0
Anti-lactoferrin (unabsorbed)	Lactoferrin, 1 μg/ml	274 ± 13	+6
Anti-lactoferrin (absorbed with lactoferrin)**	CIF-Crude	124 ± 15	−52¶
Anti-lactoferrin (absorbed with lactoferrin)**	CIF-Purified	113 ± 7	−56¶
Anti-lactoferrin (absorbed with lactoferrin)**	Lactoferrin, 1 μg/ml	108 ± 6	−58¶
Anti-lactoferrin (preincubated with transferrin)**	CIF-Crude	265 ± 8	+3
Anti-lactoferrin (preincubated with transferrin)**	CIF-Purified	250 ± 10	−3
Anti-lactoferrin (preincubated with transferrin)**	Lactoferrin, 1 μg/ml	248 ± 13	−4

* Rabbit IgG anti-human lactoferrin; the antiserum (absorbed and unabsorbed) had no effect on the assay.

‡ One part antiserum (10^{-1} dilution) to three parts test material were incubated for 2 h after a 2 h absorption with lactoferrin or transferrin and then assayed.

§ All spontaneous aggregates > 3 cells (colonies and clusters) are shown but results were similar for spontaneous colonies (>50 cells aggregate) alone.

‖ Crude CIF is initial mature granulocyte extract and purified CIF is extract subjected to DEAE Sephadex chromatography, sodium dodecyl sulfate polyacrylamide gel electrophoresis, and isoelectric focusing as in reference 6.

¶ Significant change, $P < 0.05$.

** 25 μg Lactoferrin were incubated with 0.5 ml of a 1/10 dilution of anti-lactoferrin because a titer of 500 μg/ml yields 100% absorption on the basis of Sewell test (personal communication, Dakopatts, Denmark). As a control, 25 μg transferrin was incubated with 0.5 ml of 1/10 dilution of anti-lactoferrin. When 15 μg lactoferrin were incubated with 0.5 ml of a 1/10 dilution of anti-lactoferrin, it did not absorb out the effect of the anti-lactoferrin which was still able to completely inactivate the inhibitory effects of lactoferrin and CIF.

Specificity Controls

EFFECT OF ANTI-LF AND OTHER NONRELATED ANTISERA. As shown in Table II, the purified immunoglobulin fraction of rabbit anti-human LF (at 10^{-1} and 10^{-2} dilutions) inactivated the effect of the crude and purified CIF, whereas a 10^{-1} dilution of the purified immunoglobulin fraction of rabbit anti-human TF had no influence on CIF. Rabbit anti-LF also inactivated the influence of LF on CSA production (Table III). The influence of anti-LF on CIF was blocked by prior absorption of the anti-LF with neutralizing concentrations of LF, but TF did not block the inactivation of CIF by rabbit anti-LF. Goat anti-human LF antiserum at a 10^{-3} dilution inactivated the CSA inhibitory effects of 10^{-6} μg LF and a 10^{-2} dilution of the purified CIF, whereas a 10^{-4} dilution of goat anti-LF did not inactivate 10^{-6} μg LF but partially inactivated 10^{-10} μg LF (data not shown). Normal goat serum did not inactivate LF or CIF.

In further attempts to determine the specificity of inactivations, various other

TABLE IV

Human Lactoferrin and CIF do not Inhibit Erythropoietin-Dependent Bone Marrow Erythroid Colony Formation

Test material	CFU-e	%Δ*	BFU-e	%Δ*
Human bone marrow cells				
Medium	130 ± 19		36 ± 2	
Native lactoferrin, 10^{-7} M	132 ± 6	+1	36 ± 2	0
Saturated lactoferrin, 10^{-7} M	119 ± 12	−8	36 ± 3	0
Purified CIF	119 ± 7	−8	35 ± 4	−3
Crude CIF	132 ± 7	+1	33 ± 3	−8
Intact PMN‡, 1 × 10^6 cells	110 ± 11	−15	35 ± 1	−3
BDF mouse bone marrow cells				
Medium	85 ± 9		26 ± 2	
Native lactoferrin, 10^{-7} M	79 ± 5	−7	25 ± 2	−4
Saturated lactoferrin, 10^{-7} M	83 ± 6	−2	25 ± 2	−4
Purified CIF	83 ± 9	−2	23 ± 3	−12
Crude CIF	73 ± 4	−14	25 ± 2	−4
Intact PMN, 1 × 10^6 cells	103 ± 8	+21	24 ± 2	−8
C57 BL/6 Mouse bone marrow cells				
Medium	86 ± 5		23 ± 1	
Native lactoferrin, 10^{-7} M	90 ± 5	+5	22 ± 3	−4
Saturated lactoferrin, 10^{-7} M	81 ± 7	−6	23 ± 2	0
Purified CIF	79 ± 4	−8	22 ± 2	−4
Crude CIF	80 ± 4	−6	22 ± 2	−4
Intact PMN, 1 × 10^6 cells	79 ± 6	−8	22 ± 2	−4

* None of the changes were significant, $P > 0.05$.

‡ PMN, polymorphonuclear neutrophils = mature granulocytes.

antisera were tested. None of the following antisera inactivated LF or CIF: anti-human Ia 1/40, anti-human β_2-microglobulin (10^{-1}), anti-human α_2-macroglobulin (10^{-1}), anti-human IgA (10^{-1}) and anti-human complement (C_3) (10^{-1}).

EFFECT OF ANTI-LF ON OTHER INHIBITORS OF CFU-C WHICH DO NOT SUPPRESS CSA PRODUCTION. In further specificity tests, rabbit anti-LF had no influence on inhibitory activities which can be distinguished from CIF by their cells of derivation and action. One, termed leukemia-associated inhibitory activity (LIA), is derived from leukemic bone marrow and blood cells and inhibits colony formation by normal CFU-c but not leukemic CFU-c (34, 35). The other, neutropenia-associated inhibitory activity (NIA), is derived from bone marrow and blood cells of patients with neutropenia of varying etiologies and inhibits colony formation of normal, leukemic and neutropenic CFU-c (36). LIA and NIA do not influence CSA production (34, 36).

EFFECT OF LF AND CIF ON ERYTHROID COLONY FORMATION. CIF, LF, and intact mature granulocytes were assayed for their influence on erythropoietin-dependent CFU-e and BFU-e cells. As shown in Table IV, CFU-e and BFU-e from human marrow and BDF and C57Bl/6 mouse marrow were not inhibited. These results have been repeated on three other human marrow samples and one other mouse sample.

EFFECT OF LF ON MURINE REBOUND GRANULOPOIESIS AND CSA PRODUCTION IN VIVO. Having demonstrated that LF was identical to CIF in its in vitro action, it was important to substantiate this finding in vivo. Thus, a recently published in vivo assay which depends on rebound granulopoiesis to detect inhibition by administered

TABLE V

*Inhibition of Rebound Granulopoiesis in Cyclophosphamide-Treated Mice by Lactoferrin**

Test material	Bone marrow CFU-c; no added CSA‡	%Δ	Bone marrow CFU-c; added CSA‡	%Δ
CD_1 Mice				
McCoy's medium	345 ± 85	–	3,007 ± 762	–
Transferrin	555 ± 122	+60	2,258 ± 682	−25
Lactoferrin	87 ± 33	−70§	613 ± 225	−80§
C57Bl/6 Mice				
McCoy's medium	ND‖	–	8,422 ± 525	–
Transferrin	ND	–	ND	–
Lactoferrin	ND	–	4,853 ± 871	−42§

* CDF_1 and C57Bl/6 mice were pretreated with 200 mg cyclophosphamide/kg body wt on day 0. Each mouse then received two i.v. injections (total of 1.2 ml) each on days 1 and 2. Each 0.1 ml = 1 μg lactoferrin or transferrin Mice were sacrificed on day 3 ≅12 h after the last injection. Results are given as absolute number of spontaneous or stimulated colony forming cells per femur and expressed as mean ± 1 SEM × 10^{-1} for five mice per group.

‡ No added CSA and added CSA refer to the cultures after cells are removed from the mice.

§ %Δ, Percent significant change ($P < 0.05$) from McCoy's medium.

‖ ND, Not done.

TABLE VI

Colony-Stimulating Activity in Medium Conditioned by Heart and Lung of Control, Lactoferrin- and Transferrin-Treated Cyclophosphamide Mice

Conditioned medium source	Mouse strain	Treatment of conditioned media	Colony-stimulating Activity* of mice treated with		
			Media	Transferrin	Lactoferrin
Lung	CD_1	Undialyzed	128 ± 9	120 ± 13 (−6)‡	40 ± 3 (−69)§
	CD_1	Dialyzed	174 ± 10	163 ± 15 (−6)	25 ± 3 (−86)§
	C57Bl/6	Undialyzed	145 ± 20	–	51 ± 6 (−65)§
	C57Bl/6	Dialyzed	124 ± 10	–	25 ± 3 (−80)§
Heart	CD_1	Undialyzed	68 ± 5	82 ± 11 (+21)	16 ± 4 (−76)§
	C57Bl/6	Undialyzed	73 ± 12	–	10 ± 5 (−86)§

* Colony-stimulating activity expressed as number of CFU-c ± 1 SEM/1 × 10^5 mouse bone marrow cells stimulated by 0.1 ml of a 1:2 dilution of conditioned media.

‡ Number in parentheses designates the percent change from media control.

§ Percent significant change ($P < 0.05$) from media control.

CIF (5) was utilized to test the in vivo effect of LF. Three days after i.p. injection of 200 mg cyclophosphamide/kg body wt, mature granulocytes are depleted from mice whereas absolute numbers of CFU-c and net endogenous CSA production are increased in the bone marrow (5). Administration of 20 μg human LF during the 1st and 2nd days after cyclophosphamide dampened rebound granulopoiesis in the

marrows of CD_1 and C57Bl/6 mice as assessed on the 3rd day after cyclophosphamide (Table V). LF substantially decreased total marrow CFU-c numbers as detected by adding a source of exogenous CSA to the marrow cells in culture. Inhibition of marrow CFU-c numbers was more pronounced in CD_1 (80%) than in C57Bl/6 mice (42%). Net endogenous marrow CSA production, as demonstrated in cultures without exogenously added CSA, was also substantially reduced. 20 μg of TF did not have a significant influence in these assays. Heart and lung cells removed from both mouse strains injected with LF produced 65–86% less CSA than control mice injected with McCoy's media or TF (Table VI). This was substantiated by assaying dialyzed as well as undialyzed conditioned media.

Discussion

It has been suspected that LF might have activities in addition to its capacity to act as a bacteriostatic agent (37). We have now presented evidence indicating that LF is identical to CIF and as such has the additional capacity to regulate CSA production in vitro and granulopoiesis in vivo.

Three experimental approaches were used to identify LF as the granulocyte CIF: the first was to demonstrate that LF acted in vitro and in vivo in a manner analogous to CIF; the second was to demonstrate that the inhibitory activity was localized in the same region after separation of LF and CIF by isoelectric focusing; and the third was to demonstrate that anti-LF antisera could inactivate the action of both CIF and LF.

LF, as reported earlier for CIF (2), and as documented here, inhibits CSA production by interacting with monocytes and marcrophages but does not decrease the sensitivity of the granulocyte progenitor cells (CFU-c) to CSA nor does it inactivate CSA. Significant inhibition of CSA production was attained by 10^{-14} M native LF (8% iron saturated). This effect was dependent on the iron saturation of LF molecules. Apo-LF was only minimally active at concentrations $>10^{-6}$ M whereas fully iron-saturated LF samples were active at 10^{-17}-M concentrations. The greater inhibitory activity noted with increased iron saturation of LF probably reflects further binding of lactoferrin to monocytes and macrophages. The capacity of LF to bind to macrophages is enhanced by iron saturation of the molecules (21, 22), a phenomenon believed to result from a conformational change of the protein upon iron binding (16, 21). Failure to reduce CSA production to zero levels is most likely related to the fact that monocytes and macrophages contain performed CSA (2) and that already formed but not yet released CSA is not affected by LF. The inhibitory effect of LF was fully reversed by bacterial LPS if LPS was added simultaneously with the LF, and partially reversed if LPS was added 24 h after LF. The mechanisms of these interactions are not clear but may relate to the iron-binding capacity of LPS (38, 39) because it has been reported that 1 mg LPS binds 4.8–5.2 μg of iron (38). It has been postulated that mature granulocytes limit CSA production by eliminating bacteria (40), however, mature granulocytes in the presence of bacterial products and in response to phagocytic challenge release large quantities of their intracellular contents of LF (22, 41) and suppression of CSA production in these circumstances is probably mediated via LF.

The interesting correlation between iron content and activity illustrates how one molecule can have two distinct but closely related functions. LF has bacteriostatic

and bacteriocidal activity only in the apo or native form (32, 42–46) whereas fully saturated LF is most effective and apo-LF is least effective in suppressing CSA production. Each mature granulocyte (polymorphonuclear neutrophil) contains 3–6 pg of LF (14, 47–49); this implies that there is enough LF in one mature granulocyte to inhibit CSA production of >200,000 monocytes by >60%. If, as suggested earlier, neutrophils act as exocrine cells (50) a regulatory mechanism of granulopoiesis can be envisaged as follows: native LF contained in the secondary granules of mature granulocytes (47, 51) leaves the cell, possibly in response to specific stimuli; and with an iron association constant of 3×10^{38} (16) binds iron from the serum, thus facilitating its capacity to bind to specific receptor sites on monocytes and macrophages. This then results in greater suppression of CSA production. It has been calculated that milligram quantities of LF are produced per day by granulocytes (52). This figure, added to the specific activity of LF in vitro and the microgram quantities needed for in vivo activity argue strongly for a physiological role for LF in the regulation of granulopoiesis.

Further studies of the mechanisms involved in this regulatory process are in progress. The identification of CIF as LF allows for more detailed studies in vitro and in vivo using purified and labeled molecules. With these molecules it will be possible to characterize the postulated role of LF in cell traffic (7, 8). In addition, it will now be possible to define more precisely the nature of feedback abnormalities associated with myelogenous leukemia and myelodysplasia. We have shown that CSA-producing cells of patients with chronic myelogenous leukemia are less responsive than normal to suppression by CIF (3). This phenomenon which may relate to the iron-binding capacity of patient LF or to altered or deficient LF-membrane receptors on patient monocytes and macrophages is under investigation.

Summary

Lactoferrin (LF), the iron-binding protein present in the specific granules of mature granulocytes has been identified as colony inhibitory factor (CIF) which suppresses granulocyte—macrophage colony stimulating activity (CSA) production by monocytes and macrophages in vitro and rebound granulopoiesis in vivo. Separation of LF and CIF by isoelectric focusing confirmed that the regions of inhibitory activity corresponded in both to a pH of ≅6.5. In addition, the purified immunoglobulin fraction of rabbit anti-human LF antiserum, but not rabbit anti-transferrin (TF), inactivated the capacity of LF and CIF to inhibit CSA production, an effect blocked by prior incubation of anti-LF with neutralizing concentrations of LF. Suppression of CSA production correlated with the iron-saturation of LF; apo-LF (depleted of iron) was only active at concentrations $>10^{-7}$ M, native LF (8% iron saturated) was active at 10^{-15} M, and fully iron-saturated LF inhibited at 10^{-17} M. Suppression of CSA production occurred within a ½-h preincubation period with human blood monocytes but was reversed by bacterial lipopolysaccharide (LPS). This reversal was dependent on the relative concentrations of LF to LPS. Serum TF, a biochemically similar iron-binding protein which is antigenically distinct from LF, was only minimally active at concentrations $>10^{-6}$ M. LF did not inhibit exogenously stimulated human granulocyte and macrophage colony-forming cells or erythropoietin-dependent human or murine erythroid colony- or erythroid burst-forming cells. Microgram quantities of LF acted in vivo to inhibit rebound granulopoiesis and CSA production in CD_1 and

C57Bl/6 mice pretreated with cyclophosphamide. These results strongly implicate LF as a physiological regulator of granulopoiesis.

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