

## IDENTIFICATION OF LEUKEMIA-ASSOCIATED INHIBITORY ACTIVITY AS ACIDIC ISO-FERRITINS

### A Regulatory Role for Acidic Isoferritins in the Production of Granulocytes and Macrophages\*

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Myelopoiesis is a dynamic process regulated by stimulatory and inhibitory feedback mechanisms. Aberrations in these regulatory interactions have been detected with cells from patients with leukemia, lymphoma, and myeloproliferative and myelodysplastic disorders (1). We have described *in vitro* and *in vivo* cellular interactions that may constitute the basis for the suppression of normal hematopoiesis occurring during leukemia, and that may be involved in the progression of the disease (1-7).

A synthesis (S)-phase specific inhibitory activity against normal colony-forming unit granulocyte-macrophages (CFU-GM)<sup>1</sup> is produced by bone marrow, spleen, and blood cells from patients with acute and chronic myeloid and lymphoid leukemia, lymphoma, and "preleukemia" (2, 3, 5). This activity was not detected in bone marrow and blood cells of normal donors and had been termed leukemia-associated inhibitory activity (LIA; 1-6). LIA derives from cells characterized as non-T, non-B lymphoidlike cells, or promonocytes, with Fc receptors (2, 5). Greater concentrations of LIA are found during acute leukemia (newly diagnosed and untreated, or on therapy but not in remission) than during chronic leukemia (2, 3). Remission of acute leukemia is associated with low levels of LIA (3, 6). An LIA-like substance has also been detected in bone marrow, spleen, and thymus cells from BALB/c mice infected with Abelson virus (7) and in mice infected with Friend virus (8; and L. Lu and H. E. Broxmeyer, unpublished observations). In contrast to its action on normal CFU-GM, LIA is not effective in suppressing the growth of CFU-GM from many patients

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<sup>1</sup> *Abbreviations used in this paper:* ALL, acute lymphoblastic leukemia; AML, acute myelogenous leukemia; CFU, colony-forming unit; CMC, carboxymethyl cellulose; CML, chronic myelogenous leukemia; CSF, colony stimulatory factor; GM, granulocyte macrophage; IEF, isoelectric focusing; LAI, leukemia-associated inhibitor; LIA, leukemia-associated inhibitory activity; NAIA, neutropenia-associated inhibitory activity; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

with leukemia and from BALB/c mice infected with Abelson virus and mice infected with Friend virus (1-8; and unpublished observations). LIA may thus confer a proliferative advantage to abnormally responsive progenitor cells.

We have been interested recently in the association of iron-binding proteins with cells of the myeloid and lymphoid systems (9-12) and have described a role for lactoferrin in the regulation of myelopoiesis (9, 10). We have noted now that LIA is similar in many respects to a subclass of ferritins that are also iron-binding proteins. LIA and ferritin are glycoproteins with high molecular weights (13-15) and similar isoelectric focus points (13, 16, 17). Both are elevated during acute and chronic leukemia and decreased during the remission of acute leukemia (16-18). Ferritin is considered the main storage protein for iron in mammalian cells and is thought to play a role in iron metabolism (14, 15). Ferritin can be separated into isoforms (16, 17, 19, 20), and there is evidence that isoferritins are composed of two types of subunits differing in charge and size (15-17, 19-22). The H subunit has an approximate molecular weight of 21,000 and is high in ferritin associated with heart tissue, and the L subunit has an approximate molecular weight of 19,000 and is high in ferritin associated with liver tissue. The acidic isoferritins are considered to be composed mainly of H subunits, and the most basic isoferritins appear to be composed of homopolymers of L subunits. The possible association of acidic isoferritins with tumor cells and their value in the prognosis and diagnosis of cancer have been the subject of conflicting reports (16, 17, 19, 23, 24). However, relatively little evidence has been presented that suggests that ferritin may have a role other than as a storage molecule for iron (25-27). Iron-depleted ferritin has been noted in human blood leukocytes and serum from normal donors and patients with leukemia (28); in this paper, we identify LIA as acidic isoferritins that do not appear to require iron for their activity. Evidence is given for the role of acidic isoferritins as normal inhibitory regulators of myelopoiesis, a role that is distinct from that of lactoferrin. This is, we believe, the first clear demonstration that the acidic and basic isoferritins can be distinguished in terms of biological function.

### Materials and Methods

*Source of LIA.* LIA was isolated from more than 5,000 samples of extracts of nucleated bone marrow, spleen, and blood cells collected over a 5-yr period from more than 1,000 different patients (Memorial Hospital, New York) with acute and chronic leukemia, as described previously (13).

*Source of Ferritin.* Ferritin was isolated from human spleen, liver, heart, and placental tissue. Spleen tissue was obtained from normal individuals undergoing either emergency splenectomy for a punctured spleen (Brooklyn Jewish Hospital, New York), from fatally injured persons whose kidneys were to be used for transplantation purposes (University of Heidelberg, Germany), from a patient with chronic myelogenous leukemia undergoing splenectomy as part of the treatment protocol (Memorial Hospital, New York), and from patients with Hodgkin's disease after staging laparotomy (Städtische Krankenanstalten, Darmstadt, Germany). Normal human heart and liver tissues were obtained post mortem from the University of Heidelberg, Germany, and normal human placenta ferritin was a gift from Dr. Bohn, Behringwerke A. G. Marburg, Germany. Ferritin was isolated from the above tissues immediately upon arrival or after storing the tissues at  $-20^{\circ}\text{C}$ .

*Isolation of LIA.* Pooled samples of cell extracts were isolated by a sequence of procedures as described previously (13). After ultracentrifugation, Sephadex G-200, and carboxymethyl cellulose (CMC), the pooled active fractions from CMC had a specific activity of  $6.7 \times 10^{12}$  U/1 mg protein (13) and were further isolated by either sodium dodecyl sulfate-polyacrylamide

gel electrophoresis (SDS-PAGE) or concanavalin A-Sepharose. The samples were applied to the SDS-PAGE gradient (5–20%) slab gel, pH 8.8, with 3% stacking gel (pH 6.8) without heating the sample mixtures. In some experiments, and where noted specifically in the text, the sample mixtures were heated at 100°C for 10 min to reduce the samples. Molecular markers included horse ferritin (500,000 mol wt; Sigma Chemical Co., St. Louis, Mo.), phosphorylase b (94,000 mol wt), human milk lactoferrin (76,000 mol wt; Metallo Protein Laboratories, Ltd, Windsor, Canada), bovine serum albumin (68,000 mol wt), ovalbumin (43,000 mol wt), carbonic anhydrase (29,000 mol wt), trypsin inhibitor (20,100 mol wt), hemoglobin (16,000 mol wt) and  $\alpha$ -lactalbumin (14,000 mol wt) from Pharmacia Fine Chemicals Inc., Piscataway, N. J. Active fractions from the CMC column or from SDS-PAGE were analyzed by analytical (thin-layer) or preparative isoelectric focusing (IEF).

*Isolation of Ferritin.* Ferritin was isolated at Sloan-Kettering Institute, New York, or at the University of Heidelberg, Germany. The tissue was cut into small pieces and, in the case of macroscopically involved spleens from patients with Hodgkin's disease, the nodes were separated and the nodal and non-nodal tissues were isolated. The tissues were homogenized in a Waring blender (Waring Products Div., New Hartford, Conn.) with a volume of distilled water equivalent to twofold the weight of the tissue, brought to 75°C for 15 min, and then cooled rapidly in an ice bath. Coagulated protein was removed by filtration or by centrifugation at 2,500 *g* for 20 min. The pH of the filtrate was adjusted to 4.6 and the supernate was adjusted to pH 5.5 after centrifugation. The filtrate was placed in an ice bath for 1 h and then centrifuged at 12,000 *g* for 20 min. In both cases, the filtrate and supernate were then brought to pH 6.5 with 0.2 M/1 liter  $K_2PO_4$ ;  $(NH_4)SO_4$  was added to half saturation. After 48 h at 4°C, the insoluble fraction from the filtrate was collected by centrifugation and dialyzed against glass-distilled water (4 × 2 liter vol) (29, 30). Ferritin isolated from the spleen of one patient with chronic myelogenous leukemia and one normal donor were then isolated by Sephadex G-200, CMC, SDS-PAGE, analytical and preparative IEF, and concanavalin A-Sepharose as described for the isolation of LIA. In one experiment, the insoluble fraction after half saturation with  $(NH_4)SO_4$  from a portion of a sample from the spleen of a patient with chronic myelogenous leukemia (CML) was redissolved in a solution containing 5 g of  $3 CdSO_4 \cdot 8H_2O$ /100 ml. This was left at 4°C for 48 h and the resulting crystals were collected by centrifugation at 2,500 *g* and dialyzed before further isolation by Sephadex G-200 and CMC. Other sources of ferritin (heart, N = 4; Hodgkin's spleen, N = 8; normal spleen, N = 3; normal liver, N = 3) were subjected to column chromatography on Sepharose 6B (Pharmacia Fine Chemicals, Uppsala, Sweden) after half saturation with  $(NH_4)SO_4$ . Optical density was measured at 280 nM and the peak fractions were concentrated with a UM 10 filter (Amicon Corp., Dusseldorf, Germany). The normal heart, Hodgkin's spleen, normal spleen, and normal liver ferritin were passed through an immunoabsorbent column consisting of Sepharose 4B coupled to human serum antibodies previously absorbed with human or liver ferritin, to absorb out contaminating proteins. Human placental ferritin was isolated by Dr. Bohn as described previously (31). A single sharp precipitation line was found in the peak fractions containing ferritin in double immunodiffusion studies in agarose with rabbit anti-human Hodgkin's spleen ferritin serum, which recognizes both acidic and basic isoferritins. The final preparations did not react immunologically with anti-human serum that did not contain antibodies to ferritin. Protein concentrations were determined by the ninhydrin method (32) and/or the method of Lowry et al. (33). Ferritin iron was checked qualitatively after SDS-PAGE with 2% (wt/vol) potassium ferrocyanide in 3% trichloroacetic acid, or quantitatively by atomic absorption spectrophotometry at 248.8 nm (305B; Perkin-Elmer Corp., Norwalk, Conn.) and the purity of ferritin was confirmed by gel diffusion and radioimmunoassay as described below.

*Preparation of Antisera to Ferritin.* Rabbits were immunized with either crude or purified human spleen ferritins. The first injection was given in Freund's complete adjuvant. Each rabbit received 1 mg of protein in multiple intramuscular sites at intervals of 4 wk over a period of up to 12 mo. Blood was collected 7–8 d after each injection and the serum was stored at –20°C. Antisera against human heart ferritin were obtained by immunizing goats with the purified heart ferritin in complete Freund's adjuvant intradermally at multiple sites at 4-wk intervals over a period of 4–6 mo. Antisera were purified by an immunoabsorbent column consisting of normal human serum that was previously absorbed with human ferritin and

immobilized in cyanogen bromide-activated Sepharose 4B. To obtain antisera with a greater degree of specificity for either the acidic or basic isoferritins, aliquots of goat anti-human heart ferritin or rabbit anti-human Hodgkin's spleen ferritin serum were purified by immunoabsorption. Acidic or basic isoferritins prepared by preparative isoelectric focusing were coupled to cyanogen bromide-activated Sepharose 4B beads (34).

Rabbit anti-HeLa cell ferritin (unabsorbed) (16) was generously supplied by Dr. J. W. Drysdale (Tufts University School of Medicine, Boston, Mass.). Antiserum against the H or L subunits from human liver ferritin (21, 22) was generously supplied by Dr. I. Listowsky (Einstein Medical Center, Bronx, N. Y.), and purified rabbit immunoglobulin fractions to human spleen ferritin were purchased from Dako Immunoglobulins Ltd. (Accurate Chemical and Scientific Corp., Hicksville, N. Y.).

Specificity of the antisera was checked by double immunodiffusion in 1.0% agarose gels in barbitone buffer, pH 8.6, using human spleen and heart ferritins and LIA as antigens, and by radioimmunoassay.

*Radioimmunoassay of Ferritin.* Two assay systems were used. In one assay, the purified ferritin was labeled and competed in a two-step procedure with test material and standards for antibody binding sites. This is referred to as the labeled antigen assay. In the other assay, purified antibodies to ferritin, isolated as described above, were used in a three-step procedure in which the test material and standards were added in the second step and incubated for 24 h at 4°C; the tubes were washed and the labeled antibodies were added in the third step. This is referred to as the labeled antibody assay.

*Iodination.* Purified ferritin and purified ferritin antibodies were trace-labeled with  $^{125}\text{I}$  by the chloramine T method (35). 2 mCi of  $\text{Na } ^{125}\text{I}$  (New England Nuclear, Boston, Mass.) were brought up to 100  $\mu\text{l}$  in 0.4 M borate buffer, pH 8.0. 50  $\mu\text{l}$  of  $\text{Na}[^{125}\text{I}]$  was added to 5  $\mu\text{g}$  of ferritin in a siliconized glass vial. 10  $\mu\text{g}$  chloramine T in 10  $\mu\text{g}$  of borate buffer was added to the vials and gently agitated for 15 s. The reaction was terminated by adding 15  $\mu\text{l}$  of sodium metabisulfite in 15  $\mu\text{l}$  of borate buffer. A Sephadex G-200 column (0.5  $\times$  0.5 cm) was equilibrated with borate buffer and pretreated with bovine serum albumin. Bovine serum albumin (25 mg in 500  $\mu\text{l}$  of buffer) was added as a "carrier," and bound and free  $^{125}\text{I}$ -ferritin was then separated by gel chromatography.

The immunoreactive fractions were pooled with 0.06 M barbitone buffer, pH 9.6, containing 250 mg/100 ml bovine serum albumin. The small amount of solution remaining in the "reaction tube" was used to assess the percentage of iodination by the trichloroacetic acid precipitation method. Immunoreactivity was assessed by the ability of the iodinated or noniodinated protein to bind to the antibody coated vials.

*Antibody Coating of Tubes.* Polypropylene tubes (Walter Sarstedt, Inc., Princeton, N. J.) were coated with ferritin antibodies by a modification of the method of Catt and Tregear (36). Antibodies were diluted  $2 \times 10^{-4}$  in 0.06 M barbitone buffer, pH 9.6, as determined by a previous titration curve. Preliminary studies showed that this dilution gave 85% of the maximum binding capacity after 24 h. 1 ml of diluted antibody was added to each vial and the vials were left at 4°C for 24 h. The vials were washed three times with 0.9% NaCl, once with 0.9% NaCl containing 1 g/100 ml of bovine serum albumin (to saturate nonantibody-coated binding sites), and three times with 2 ml of normal saline. The coated tubes were kept at 4°C for up to 1 wk.

*Treatments.* All treatments were performed with the most purified preparations of LIA containing specific activities of  $6.7 \times 10^{12}$ – $1.0 \times 10^{14}$  U/mg protein (13) and with normal and CML-spleen ferritin which were isolated by CMC, SDS-PAGE, and IEF.

*Enzymatic Digestion.* The enzymes shown in Table III were purchased from Sigma Chemical Co. and were used as described previously (13).

*Periodate Oxidation.* The test sample (100  $\mu\text{l}$ ) was added to 2  $\mu\text{l}$  of 0.01 M potassium periodate in 0.1 M sodium acetate-HCl, pH 5.0, and the solution was incubated in the dark for 4 h at 4°C. The reaction was stopped by the addition of 200  $\mu\text{l}$  of 50% sucrose per 2 ml of sample. The oxidized samples were dialyzed against 2 mM sodium phosphate buffer (pH 7.0).

*Temperature Treatments.* Samples were exposed to 37°C for 5 d, 56°C for 30 min and 75°C for 5–20 min.

*Iron Depletion.* Samples were iron depleted by reduction. 1 mg of ferritin was mixed with a

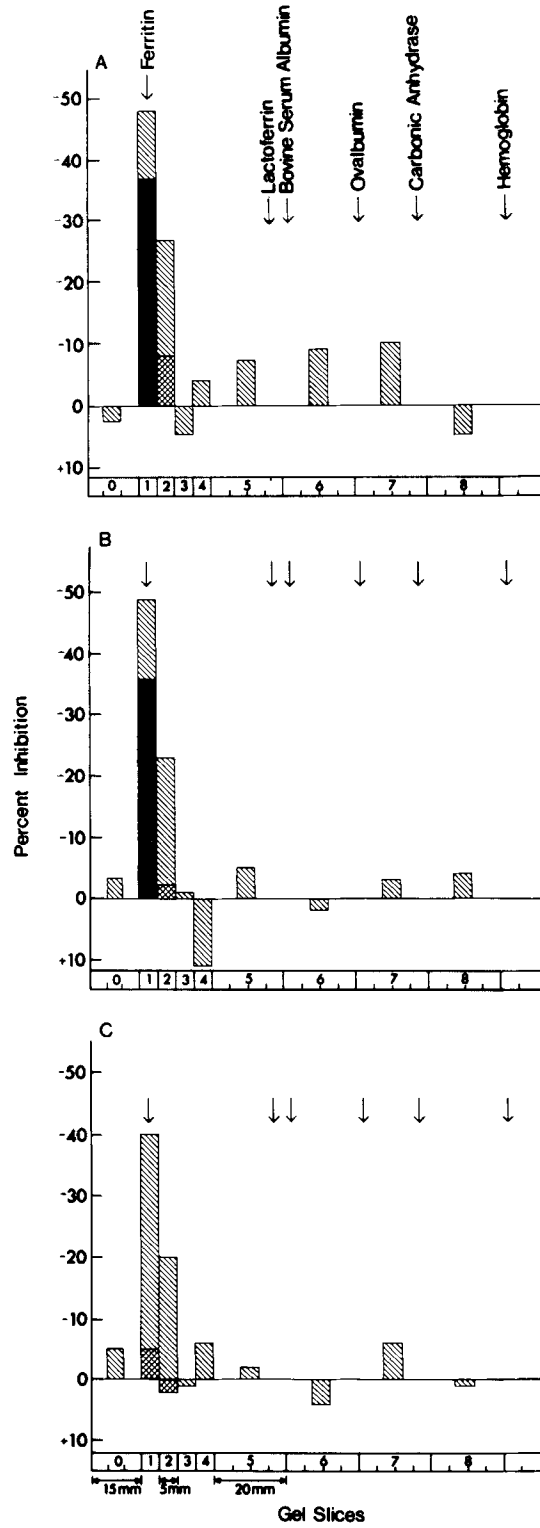


FIG. 1. The comparative isolation of inhibitory activity from LIA (A), CML-spleen ferritin (B), and normal spleen ferritin (C) by SDS-PAGE. Absorbance is not shown but was detectable only in gel fraction 1 for both CML spleen and normal spleen ferritin. Control colony and cluster numbers ranged from 210 to 450 in individual experiments. ▨, 10<sup>0</sup> dilution; ▩, 10<sup>-1</sup> dilution; ■, 10<sup>-5</sup> dilution.

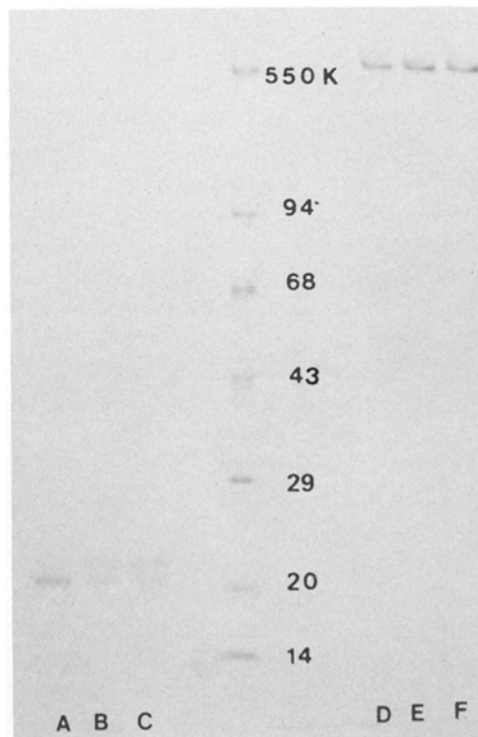


FIG. 2. The comparative migration of protein bands from LIA and CML-spleen ferritin after reduction (A-C) and under nonreducing conditions (D, E, and F) after SDS-PAGE. The numbers signify the approximate molecular weights of the markers. A, reduced CML-spleen isoferritin (pH, 4.7); B, reduced CML-spleen acidic isoferritin (pH, 5.8); C, reduced LIA; D, LIA (CMC); E, LIA (pH, 4.7); F, CML-spleen acidic isoferritin (pH, 4.7).

200- $\mu$ M solution of reduced flavin mononucleotide plus sodium dithionite (2.0 mM) in 25 mM Tris-HCl, pH 7.4. This was further reduced by dialysis against 10 mM thioglycolic acid (37). The samples were then dialyzed against glass distilled water ( $5 \times 2$  liter vol) and iron measurements were made by atomic absorption spectrophotometry.

**Reducing Conditions of Subunit Dissociation.** Samples were mixed with 10% 2-mercaptoethanol in 0.125 M Tris buffer, pH 6.8, boiled for 10 min, and dialyzed against glass-distilled water ( $5 \times 2$  liter vol; 38).

**Assay for LIA.** Colony ( $>50$  cells/aggregate) and cluster (3-50 cells/aggregate) formation of bone marrow cells was stimulated by exogenously supplied colony stimulatory factors (CSF) as described previously (13). Plates were scored after 7 d of incubation, a time we have shown previously to be optimal for demonstration of inhibition of CFU from normal donors by LIA (2). To ensure that the scorer was unaware of the nature of the samples to be assessed for inhibitory activity, the samples were coded in  $>70\%$  of the studies by another person before being assayed. All the antisera inactivation studies and at least two complete experiments in each category were scored in this way.

**Statistical Analysis.** 3-5 plates were scored for each sample and the probability of significant differences between samples was determined by use of Student's *t* test.

## Results

**Comparative Biochemical Isolations of LIA and Ferritin.** To substantiate the biochemical similarities between LIA and ferritin (13-17), the samples were isolated by SDS-PAGE and analytical and preparative IEF. The LIA had been purified previously by

a combination of procedures, including Sephadex G-200 and CMC, and had a specific activity of  $6.7 \times 10^{12}$  U/mg protein (13). Highly purified samples of ferritin obtained from spleen tissue of a normal accident victim and from a patient with CML were also used. The samples were isolated by nonreducing SDS-PAGE. Two preliminary experiments using gel cuts (20 mm each) localized the inhibitory activity of the three samples in the region of apparent molecular weights of  $>300,000$ . A subsequent experiment using gel cuts of 10 mm each localized the inhibitory activity in an apparent molecular weight region  $>450,000$ . The graph shown in Fig. 1 demonstrates the average results of four experiments for LIA, three for CML-spleen ferritin, and four for normal spleen ferritin, in which 5-mm gel cuts were made in the molecular weight region of 300,000–550,000. From these experiments an apparent molecular weight range of  $\sim 450,000$ –550,000 (gel fractions 1 and 2) was calculated for the three samples with most of the inhibitory activity localizing in the range of 550,000 (gel fraction 1). All significant activity was lost from gel fraction 2 after a  $10^{-1}$  dilution, but plateau levels of inhibition were still present in gel fraction 1 after a  $10^{-5}$  dilution ( $P < 0.001$ ). No significant activity was noted in the region of the stacking gel (1st 15 mm, gel slices 0). Fig. 2 demonstrates the nonreduced protein bands of LIA (D, previously isolated to the stage of CMC, and E, pH 4.7 after IEF) and acidic isoferritin (pH 4.7) isolated from the spleen of a patient with CML. The three preparations comigrated with the horse spleen ferritin molecular weight marker ( $\sim 550,000$  mol wt) and inhibitory activity (50–55%,  $P < 0.001$ ) was detected only in this region of the gel. Reduction of the acidic LIA (Fig. 2 C) and acidic spleen ferritin (Fig. 2 B) by heating the sample mixture for 10 min at  $100^\circ\text{C}$  before running the samples on the gel resulted in two bands of protein detected in the molecular weight range of 20,000. For comparison, a preparation of basic isoferritin (pH 5.8) was reduced (Fig. 2 A) and one band of protein coincident with the lower molecular weight band of the other two reduced preparations was seen (Fig. 2 A). Inhibitory activity was not detected after reduction in the region of the low molecular weight bands (Fig. 2 A–C).

A profile of inhibitory activity after IEF of three samples is shown in Fig. 3. This

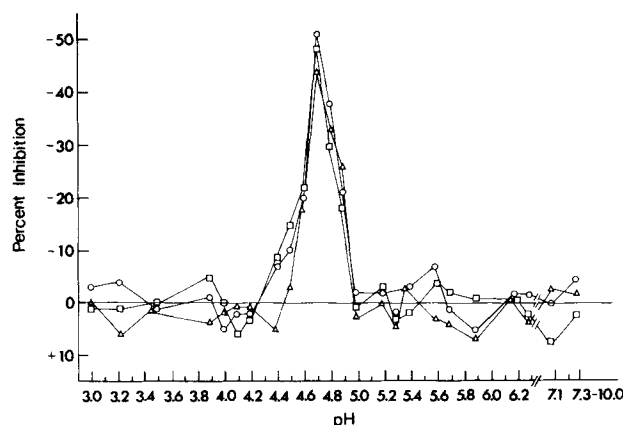


FIG. 3. The comparative isolation of inhibitory activity from LIA (O), CML-spleen ferritin (□), and normal spleen ferritin (Δ) by analytical and preparative IEF. Absorbance is not shown but was detectable in the ranges of pH 4.4 to 5.9. Control colony and cluster numbers ranged from 150 to 390 in individual experiments.

includes the average of four experiments for LIA, five experiments for CML-spleen ferritin, and two experiments for normal spleen ferritin; the results of analytical and preparative IEF that were similar are combined. Maximum activity was detected at pH 4.7 ( $P < 0.001$ ) with significant activity also noted at pH 4.6 ( $P < 0.01$ ), pH 4.8 ( $P < 0.001$ ), and pH 4.9 ( $P < 0.01$ ). Low but significant activity was noted for the CML-spleen ferritin at pH 4.5 ( $P < 0.05$ ). Bands of protein from samples of LIA (pH,  $< 5.0$ , which was first isolated by preparative IEF) and ferritin from the spleen of a patient with CML were seen after IEF in polyacrylamide gels (Fig. 4). Bands were noted in the LIA sample at pH 4.5, 4.65, 4.94, and 4.97, and in the spleen sample at pH 4.5, 4.65, 4.94, 4.97, and 5.65. Inhibitory activity in both samples was localized to the pH ranges of 4.65–4.97 with optimal inhibitory activity in the range of pH 4.65 (50–55%,  $P < 0.001$ ).

*Detection of Ferritin in Samples of LIA by Gel Diffusion.* Samples of LIA (pH  $< 5.0$ ), acidic (pH 4.9), and basic (pH 5.8), heart ferritin isolated by preparative IEF and Hodgkin's spleen ferritin (containing acidic and basic ferritins) were analyzed for immunological cross-reactivity with anti-H and -L subunit sera (32, 33), rabbit anti-Hodgkin's spleen ferritin, and goat anti-human heart ferritin sera by the Ouchterlony diffusion technique (Fig. 5). The latter two sera were used unabsorbed or after absorption with basic heart ferritin (pH 5.8). Strong precipitation lines were seen between LIA, acidic heart ferritin (F 3), Hodgkin's spleen ferritin (F 1), and the anti-H subunit sera, and the unabsorbed and absorbed rabbit and goat anti-ferritin sera (Fig. 5a–c, e, and f). Very faint precipitation lines were noted between LIA, acidic heart ferritin, and anti-L subunit antiserum (Figure 5d). Basic heart ferritin was recognized strongly by anti-L subunit antiserum (Fig. 5d) and very weakly by

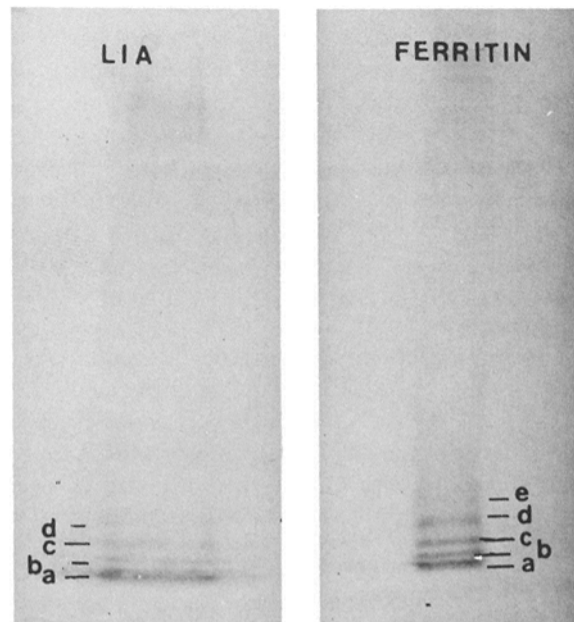


FIG. 4. The comparative migration of protein bands from LIA, (pH  $< 5.0$ ) and CML-spleen ferritin. The pI values are 4.5 (a), 4.65 (b), 4.94 (c), 4.97 (d), and 5.65 (e).



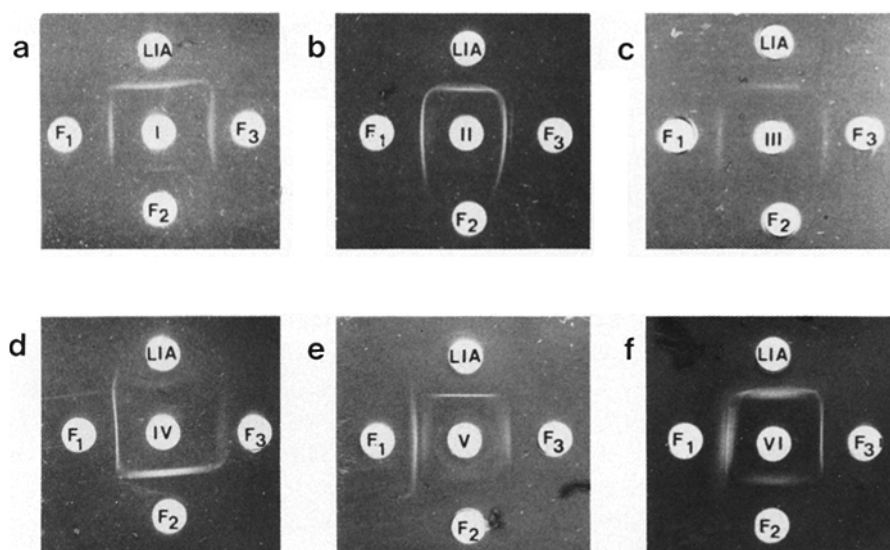


FIG. 5. Ouchterlony gel diffusion of LIA and ferritin against anti-ferritin sera. The antigens used were LIA (pH 5.0), acidic heart ferritin, pH 4.7 (F3), basic heart ferritin, pH 5.8 (F1), and Hodgkin's spleen ferritin (F2). The antisera used were: I, anti-H subunit sera (a); II, rabbit anti-Hodgkin's spleen ferritin absorbed with basic ferritin (pH 5.8; b); III, goat anti-heart ferritin absorbed with basic ferritin (pH 5.8; c); IV, anti-L subunit sera (d); V, unabsorbed rabbit anti-Hodgkin's spleen ferritin (e); and VI, unabsorbed goat anti-heart ferritin (f).

unabsorbed goat anti-heart ferritin sera (Fig. 5 f). These results demonstrate the presence of acidic isoferritins in the preparation of LIA.

*Detection of Ferritin in Samples of LIA by Radioimmunoassay.* Radioimmunological detection of ferritin was accomplished using either labeled antigen or labeled antibody (Table I). Three different unabsorbed antisera and one absorbed antiserum were used to coat the tubes. None of the antisera recognized lactoferrin or transferrin (data not shown). Detection of ferritin by radioimmunoassay has been shown by others to vary with the type of antibody used and may underestimate, especially with regards to the acidic isoferritins, the actual content of ferritin present (16). This is clearly demonstrated by the data in Table I using the labeled antigen assay. In this assay, ferritin was detected in all the samples but the rabbit anti-HeLa cell ferritin serum was more sensitive in recognizing the ferritin in LIA and the functionally active CML spleen samples. Moreover, a comparison of the two assays using unabsorbed goat anti-normal heart ferritin and unabsorbed rabbit anti-spleen ferritin demonstrated that the labeled antibody technique was more effective in recognizing the acidic isoferritins than the labeled antigen technique. Very little difference was noted between the unabsorbed goat anti-normal heart ferritin and this antiserum absorbed with basic ferritin using the labeled antibody procedure. The data in this table demonstrate that samples of LIA (pH 4.7) and CML spleen (pH 4.7) contain at least 90–95% ferritin.

*Inactivation of the Action of LIA and Ferritin by Anti-Ferritin Sera.* Six different anti-human ferritin sera were able to inactivate the inhibitory action of LIA and ferritin. The antisera used included three rabbit anti-Hodgkin's spleen ferritins, two rabbit anti-normal spleen ferritins (one of which was purchased commercially), and one goat anti-normal heart ferritin. The samples containing inhibitory activity included crude

TABLE I  
*Determinations of Ferritin Concentrations by Radioimmunoassay*

Antisera used to coat tubes	Labeled antigen			Labeled antibody		
	Unabsorbed rabbit anti-HeLa cell ferritin	Unabsorbed goat anti-normal heart ferritin	Unabsorbed rabbit anti-spleen ferritin	Unabsorbed goat anti-normal heart ferritin	Goat anti-normal heart ferritin absorbed with basic heart ferritin, pH 5.8	Unabsorbed rabbit anti-spleen ferritin
<i>LIA</i>						
CMC	68	68	24	80	83	72
SDS-PAGE	85	74	56	90	94	77
IEF (pH 4.7)	90	71	65	92	94	80
<i>Ferritin</i>						
CML-spleen (CMC)	94	84	70	87	89	76
CML-spleen, pH 4.7	95	65	65	89	90	73
CML-spleen, pH 4.9	85	84	70	92	92	75
CML-spleen, pH 5.3	80	90	85	86	85	86
CML-spleen, pH 5.5	90	90	92	83	78	89
CML-spleen, pH 5.9	97	90	94	81	75	89

Details on radioimmunoassay are given in Materials and Methods. The standards for all the assays were CML-spleen ferritin. Protein determinations were made by the method of Lowry et al (33).

LIA, LIA isolated by SDS-PAGE, LIA isolated by preparative IEF, CML-spleen ferritin, normal spleen ferritin, and normal heart ferritin. The specificity of two different antisera for the acidic isoferritins is shown in Table II. Ferritin antisera, previously absorbed with basic isoferritins on a solid-phase immunoabsorbant column whose specificity was demonstrated in Fig. 5, were still capable of inactivating the inhibitory activity of the LIA and ferritin samples. However, the ferritin antisera absorbed with the acidic isoferritins lost their inactivating capacity. As further controls, antisera were assessed for their capacity to inactivate a neutropenia-associated inhibitory activity (NAIA), which has been shown to be different from LIA (39). The above anti-human ferritin sera inactivated the inhibitory activity of the samples of LIA and ferritin but not of NAIA (data not shown).

*Comparative Physicochemical Characteristics of LIA and Ferritin.* The effect of various treatments on the activity of LIA and ferritin is presented in Table III. The action of LIA and ferritin were inactivated by treatment with trypsin, chymotrypsin, pronase, and periodate. The apparent glycoprotein nature of the inhibitory activities is consistent with the results shown in Fig. 6. The inhibitory activity in the LIA and ferritin samples bound to concanavalin A-Sepharose columns and were eluted off by  $\alpha$ -methyl mannose. Heating at 100°C under reducing conditions also inactivated the samples (Table III). The effect of the latter treatment was confirmed when no inhibitory activity was recovered from the SDS polyacrylamide gels in which the samples had been heated to 100°C before placing the sample mixtures on the gel (LIA, two experiments; CML and normal-spleen ferritin, one experiment each). Confirmation of the breakdown of the samples into subunits was apparent with the LIA and CML-spleen ferritin, which demonstrated two protein bands in the molecular weight region of ~20,000 (Fig. 2 B and C). LIA and ferritin were not inactivated by treatment with DNase, RNase, neuraminidase, lipase, and phospholipase C. The activity of LIA and ferritin was not altered by treatments yielding apparently complete iron depletion; an untreated sample of CML-spleen ferritin contained 1.34  $\mu$ g iron/ml and an untreated sample of CML-spleen acidic isoferritin (pH 4.7) contained 0.5  $\mu$ g iron/ml, and no iron could be detected in the iron-depleted samples.

TABLE II  
*Specificity of the Anti-Ferritin Inactivation of the Inhibitory Activity of Ferritin and LIA*

Antiserum to ferritin*	Antiserum absorbed with‡	Test material§	Colonies and clusters	%Δ
None	—	McCoy's medium	230 ± 8	—
None	—	Spleen ferritin	132 ± 7¶	-43
None	—	LIA-IEF	130 ± 6¶	-43
Rabbit anti-Hodgkin's spleen	Unabsorbed	Spleen ferritin	237 ± 12	+3
Rabbit anti-Hodgkin's spleen	Unabsorbed	LIA-IEF	218 ± 5	-5
Goat anti-normal heart	Unabsorbed	Spleen ferritin	222 ± 7	-3
Goat anti-normal heart	Unabsorbed	LIA-IEF	234 ± 4	+2
Rabbit anti-Hodgkin's spleen	Basic	Spleen ferritin	220 ± 6	4
Rabbit anti-Hodgkin's spleen	Basic	LIA-IEF	230 ± 9	0
Goat anti-normal heart	Basic	Spleen ferritin	234 ± 10	+2
Goat anti-normal heart	Basic	LIA-IEF	226 ± 6	-2
Rabbit anti-Hodgkin's spleen	Acidic	Spleen ferritin	125 ± 8¶	-46
Rabbit anti-Hodgkin's spleen	Acidic	LIA-IEF	135 ± 7¶	-41
Goat anti-normal heart	Acidic	Spleen ferritin	127 ± 5¶	-45
Goat anti-normal heart	Acidic	LIA-IEF	118 ± 3¶	-49

\* One part antiserum ( $10^{-2}$  dilution) to four parts test material were incubated for 1.5 h at room temperature before the assay. The antiserum (unabsorbed and absorbed) had no effect on the assay (+3 to -5 %Δ from McCoy's medium control).

‡ Absorption of the antisera is explained in Materials and Methods. Rabbit anti-Hodgkin's spleen ferritin was absorbed with either Hodgkin's spleen ferritin, pH 5.6 (basic) or pH 4.9 (acidic), and goat anti-normal heart ferritin was absorbed with either Hodgkin's spleen ferritin, pH 5.9 (basic) or pH 4.9 (acidic).

§ Spleen ferritin came from a patient with CML and contained 94% ferritin as determined by radioimmunoassay using rabbit anti-HeLa cell ferritin, and the pooled LIA after IEF (pH 4.7) contained 90% ferritin (see Table I). Preincubation with antiserum was done with test material containing  $10^{-9}$ - $10^{-12}$  M ferritin.

|| Data for colonies and clusters are shown but results for colonies were similar. Five plates were scored per datum point.

¶ Significant change,  $P < 0.0025$ .

Interestingly, although the inhibitory activity of purified samples of LIA and ferritin was extremely heat stable, crude LIA, which was not dialyzed or collected in the presence of phenylmethylsulfonyl fluoride (PMSF), a serine protease inhibitor, was very sensitive to inactivation by heat. This latter phenomenon may have resulted from contamination of the crude LIA preparations with proteolytic enzymes. It is known that PMSF stabilizes the activity of crude preparations of LIA against loss by heat treatment (2) and stabilizes ferritin molecules (40).

*Titration of the Inhibitory Activities of LIA and Ferritin.* LIA is S-phase specific in action and when assayed for its capacity to inhibit colony- and cluster-forming cells, it exhibits a titration curve of inhibition that plateaus between 30 and 60% of control values (3). A plateau curve of inhibition is noted in Fig. 7 for all the samples tested. The molarities were calculated from the protein content of the samples as determined by the method of Lowry et al. (33) and assumes a molecular weight of 550,000 for all the samples. The one sample of ferritin isolated by crystallization with  $\text{CdSO}_4$  resulted in ~50% inhibition at  $10^{-10}$  M, but was not titrated for its activity and is not included in the titration curve. CML-spleen ferritin (pH 4.7), LIA (pH 4.7), and normal heart ferritin contained the more potent activities with plateau levels of inhibition detected

TABLE III  
Comparative Physicochemical Stabilities of LIA and Ferritin

Treatment	Percentage of change ( $Y \pm 1$ SEM) from control		
	LIA* (5)‡	CML-spleen ferritin (3)§	Normal spleen ferritin (2)
None	-47 ± 4	-46 ± 3	-54 ± 4
<i>Treatments inactivating inhibitory activity</i>			
Trypsin	0 ± 2	+1 ± 3	+2 ± 2
Chymotrypsin	-1 ± 2	-3 ± 3	-2 ± 2
Pronase	-1 ± 2	-3 ± 2	-1 ± 5
Periodate	-1 ± 1	-1 ± 2	-5 ± 1
Subunit breakdown	-1 ± 2	-1 ± 4	N.D.
<i>Treatment not inactivating inhibitory activity</i>			
DNase	-44 ± 4	-47 ± 3	-51 ± 1
RNase	-46 ± 4	-45 ± 5	-53 ± 3
Neuraminidase	-49 ± 5	-46 ± 6	-47 ± 4
Lipase	-46 ± 4	-46 ± 3	-52 ± 2
Phospholipase C	-46 ± 4	-50 ± 3	-53 ± 7
Iron depletion	-44 ± 4	-46 ± 6¶	N.D.
37°C (5 d)	-49 ± 4	-42 ± 4	N.D.
75°C (20 min)	-55 ± 4**	-42 ± 4	N.D.

\* Data include results using LIA isolated by CMC, CMC and then SDS-PAGE, and CMC and then IEF (pH 4.7). LIA isolated after each purification stage was tested at least once with the above treatment protocols. The subunit breakdown and iron-depletion experiments were performed on LIA isolated by SDS-PAGE or IEF.

‡ Parentheses indicate the number of different samples subjected to the full treatment protocol. Each different sample population within a column was assayed on a different normal bone marrow.

§ Data include ferritin isolated by CMC, CMC and then SDS-PAGE, and CMC and then IEF (pH 4.7).

|| Data include ferritin isolated by CMC.

¶ Treated and untreated samples of ferritin (5.4 mg/ml each) isolated by CMC were assessed for their content of iron by atomic absorption spectrophotometry. Also, an untreated sample of CML-spleen ferritin contained 1.34 µg iron/ml, and an untreated sample of CML-spleen acidic isoferritin (pH 4.7) contained 0.5 µg iron/ml, and no iron could be detected in the iron-depleted samples.

\*\* Crude LIA (dialyzed cell extract, N = three experiments) was inactivated after treatment at 75°C for 5 min but LIA was isolated by CMC, CMC and then SDS-PAGE, or CMC and then IEF (pH 4.7), was resistant to treatment with temperatures of 75°C for 20 min.

at  $10^{-16}$ – $10^{-17}$  M, and some significant activity ( $P < 0.05$ ) still was detected at  $10^{-18}$ – $10^{-19}$  M. CML-spleen ferritin, Hodgkin's spleen ferritin, normal placental ferritin, and LIA isolated by SDS-PAGE were nearly as potent, with plateau levels of inhibition noted at  $10^{-15}$ – $10^{-16}$  M and significant activity ( $P < 0.05$ ) was still seen at concentrations of  $10^{-17}$ – $10^{-18}$  M. The effect of normal spleen ferritin fell into two categories, in which two samples were slightly less active than the CML-spleen ferritin and two other samples were only active to  $10^{-13}$ – $10^{-14}$  M. Normal liver ferritin was active to  $10^{-15}$ – $10^{-16}$  M.

*Comparative Action of LIA and Ferritin on CFU-GM from Patients with Leukemia.* LIA is not active against CFU-GM from many patients with leukemia (1, 3, 6). For this reason, crude LIA, LIA purified by SDS-PAGE, CML-spleen ferritin, CML-spleen acidic isoferritin (pH 4.7), and normal spleen ferritin at concentrations of  $10^{-10}$ – $10^{-13}$  M were assessed for their capacity to inhibit colony and cluster formation in samples of bone marrow from patients with leukemia. The data shown in Fig. 8 are divided

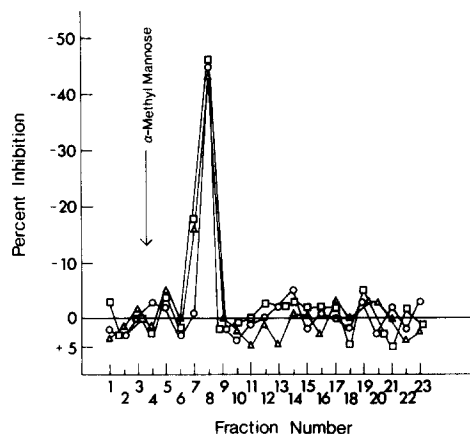


FIG. 6. The comparative isolation of inhibitory activity from LIA (○), CML-spleen ferritin (□), and normal spleen ferritin (△) by concanavalin A-Sepharose. Absorbance is not shown but was found in fractions 1 and 6 for LIA and fractions 1, 2, and 5–10 for the CML and normal spleen ferritin. Control colony and cluster numbers ranged from 310 to 450.

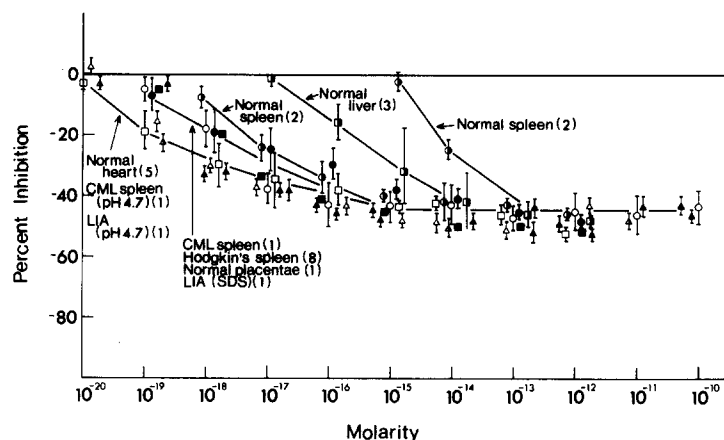


FIG. 7. Titration curve of the inhibitory activity of LIA and ferritin. Control colony and cluster numbers ranged from 110 to 450. The numbers in parentheses designate the number of different preparations used. Each sample was assayed on 1–3 different target marrow samples and the curve for the CML spleen ferritin and CML spleen ferritin (pH 4.7) was an average of the results of five marrows. □, normal heart; ■, normal placenta; ▣, normal liver; ○, CML spleen; ●, Hodgkin's spleen; ◐, normal spleen; △, CML-spleen (pH 4.7); ▲, LIA (SDS); ▴, LIA (pH 4.7).

into those samples that responded to the activities and those that did not respond. In 116 of the 118 bone marrow samples examined, the five preparations tested worked identically. Divergence between the preparations was only observed in two cases (data not shown).

### Discussion

Ferritins from mammalian tissue can be resolved into acidic and basic components by IEF, and are considered to be families of hybrid molecules consisting of different proportions of two subunits, H and L (15–17, 19–22). The more acidic isoferritins contain greater proportions of the H subunits, and the most basic isoferritins appear

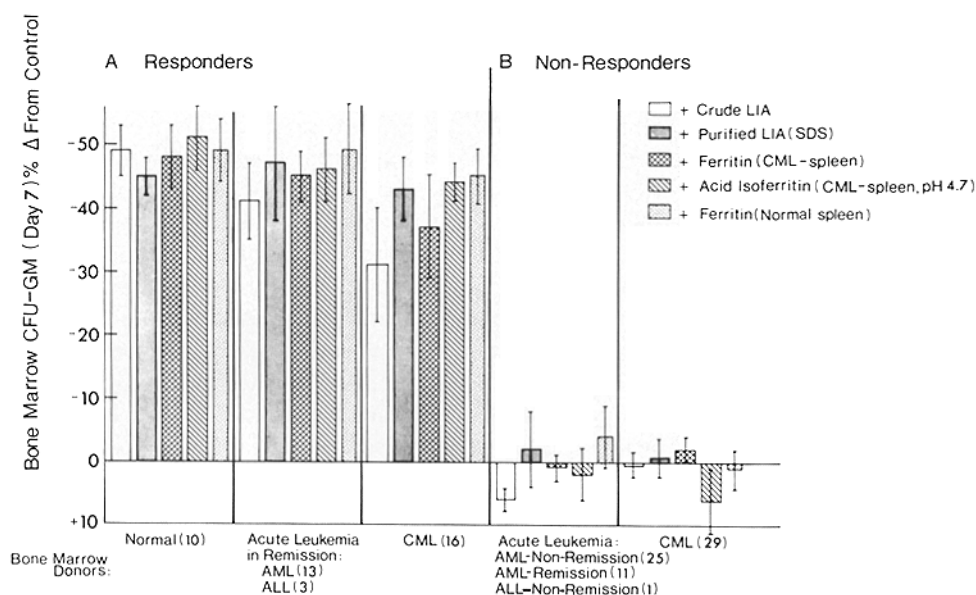


FIG. 8. Comparative action of LIA and ferritin on colony- and cluster-forming cells from patients with leukemia. Control colony and cluster numbers from the patients with leukemia ranged from 43 to 1,010 and numbers from the normal donors ranged from 150 to 380.

to be composed of homopolymers of L subunits. We have now identified LIA, a suppressor activity of normal granulocyte and macrophage progenitor cells, as acidic isoferitins. This defines a functional role for acidic isoferitins in normal and abnormal regulation of the production of granulocytes and macrophages, and is the first clear demonstration that acidic ferritins can be distinguished from basic ferritins functionally, with regard to a property different from iron uptake (41).

The identity of LIA as acidic isoferitins was established by various criteria: (a) The inhibitory activity in preparations of LIA and ferritin copurified with the protein bands of LIA and ferritin after isolation by nonreducing SDS-PAGE and with acidic isoferitins after isolation by IEF. (b) Ferritin was found in LIA samples by Ouchterlony gel diffusion using antisera that recognize acidic isoferitins and H subunits, and 90–94% of the total protein of LIA preparations was determined by radioimmunoassay to consist of ferritin. (c) Inhibitory activity characteristic of LIA was found in all ferritin preparations analyzed and the greatest amount of activity was noted in preparations enriched for acidic isoferitins. Basic isoferitins (pH >5.0) isolated by IEF were inactive as inhibitors. (d) The inhibitory activity present in LIA and ferritin preparations, but not in preparations containing NAIA, was inactivated by a battery of antisera specific for ferritin, including antisera relatively specific for acidic isoferitins. (e) The inhibitory activity in LIA and ferritin had similar physicochemical characteristics. The inactivation by periodate treatment and the binding to concanavalin A-Sepharose and elution by  $\alpha$ -methyl mannose is also consistent with LIA being an acidic isoferitin because the acidic, but not the more basic, ferritins bind to concanavalin A-Sepharose (42).

We were not able previously to detect LIA in bone marrow and blood cells from normal donors (1–3, 5, 6) but as shown here, this type of inhibitory activity was also

found in heart, spleen, placenta, and liver tissues from normal individuals. This was probably due to a combination of factors. Acidic isoferritins are elevated in leukemia and lymphoma, but are present in very low concentrations in bone marrow and blood cells and in serum from normal donors (16, 17, 19). Crude preparations of cell extracts and conditioned medium were used, and it is now apparent that proteolytic enzymes inherent in these preparations can inactivate the inhibitory activity of ferritin. Additionally, small quantities of inhibitory activity of acidic isoferritins from normal cells may have gone undetected because our assay depends on the suppression of colony formation stimulated by GM-CSF. We have now been able to detect acidic isoferritin inhibitory activity in medium conditioned by human placental cells and adherent mononuclear bone marrow and blood cells from normal donors which also contains GM-CSF (H. E. Broxmeyer and J. Bognacki, unpublished observations). The stimulatory activity of these crude preparations of conditioned medium are enhanced by 50–100% after removal or inactivation of the acidic isoferritins, by preincubation of the conditioned medium with antiserum to acidic isoferritins, or by passing the conditioned medium over Sepharose 6B columns. Although the GM-CSF-containing conditioned medium used in these studies contained acidic ferritin, the sensitivity of CFU-CM to suppression by acidic isoferritins is enhanced (e.g., 60 vs. 40% inhibition) by using GM-CSF preparations depleted of acidic isoferritins (unpublished observations).

The inhibitory activity was detected at concentrations as low as  $10^{-17}$ – $10^{-19}$  M, assuming a molecular weight of 550,000. These results bring the potency of acidic isoferritins into the range of activity noted from lactoferrin, another iron-binding glycoprotein, which at concentrations in the range of  $10^{-17}$  M inhibits the production of GM-CSF from monocytes and macrophages. It remains to be determined whether the final effect is due to a direct action on the colony-forming cell (CFU-GM), a single molecule, multiple-target hit situation, or due to a cascade of events set off by acidic isoferritin molecules.

It has been shown by others that the highest iron content is found in the most acidic isoferritins from human heart and liver, that iron concentrations in ferritin decrease with rising pI (41, 43), and that the acidic fractions take up iron more rapidly than the basic ones (41). However, in contrast to lactoferrin, which must be in an iron saturated form to bind to mononuclear phagocytes and to inhibit production of GM-CSF (9, 10), acidic isoferritins do not appear to require iron saturation for their inhibitory activity.

The relevance of acidic isoferritins as regulators of myelopoiesis *in vivo* remains to be determined, but the low concentrations needed for activity *in vitro* suggest that they may be of importance as physiological regulators, a role we have postulated previously for lactoferrin (9). The acidic isoferritins may be steady state regulators of the production of granulocytes and macrophages, but the increase of acidic isoferritin inhibitory activity during leukemia and lymphoma, and the insensitivity of "leukemia" colony-forming cells to inhibition by the acidic isoferritins, also suggest a role for these substances in the progression of leukemia. In this context, it is of interest that lactoferrin inhibitory activity is deficient or absent during leukemia and that this deficiency coincides with the presence of increased levels of acidic isoferritin inhibitory activity. This suggests that a compensatory feedback system is operating during leukemia. From *in vivo* studies with mice infected with Abelson or Friend virus and

from a correlation of *in vitro* studies with disease progression in man (1-8; and L. Lu and H. E. Broxmeyer, unpublished observations) it appears that insensitivity of leukemia CFU-GM to inhibition by acidic isoferritins precedes the increase in acidic isoferritin inhibitory activity measured against normal CFU-GM.

Another leukemia-associated inhibitor (LAI) has been reported by others (44, 45). LAI has many characteristics in common with LIA, although subtle differences have been noted. It remains to be determined whether LAI is also a population of ferritin molecules.

The identification of LIA as acidic isoferritins will make possible the more precise definition of its action *in vitro* and *in vivo*, its exact role and significance in the regulation of normal and abnormal myelopoiesis, and the characterization of the mechanisms whereby "leukemia" cells escape its action.

### Summary

Acidic isoferritins have been identified as leukemia-associated inhibitory activity (LIA), which suppresses colony and cluster formation of colony-forming unit-granulocyte macrophages from normal donors but not from patients with leukemia. LIA was detected in all the ferritin preparations tested, including ferritin isolated from normal heart, spleen, liver, and placental tissues, and from the spleens of patients with chronic myelogenous leukemia and Hodgkin's disease. Purified preparations of LIA were composed almost entirely of acidic isoferritins, as determined by immunoassay, radioimmunoassay, and isoelectric focusing. The inhibitory activity in the LIA and ferritin samples was inactivated by a battery of antisera specific for ferritin, including those prepared against acidic isoferritins from normal heart and spleen tissues from patients with Hodgkin's disease, and those previously absorbed with basic isoferritins. Antisera absorbed with acidic isoferritins did not inactivate the inhibitory activity. Separation of LIA and chronic myelogenous leukemia and normal spleen ferritin by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and isoelectric focusing confirmed that the regions of peak inhibitory activity corresponded in each to an apparent molecular weight of ~550,000 and to a pI value of 4.7. Similar physicochemical characteristics included inactivation by methods that dissociate ferritin molecules into subunits and by treatment with trypsin, chymotrypsin, pronase, and periodate. The purified preparations were extremely stable to heat treatment. The glycoprotein nature of the inhibitory activity was substantiated because it bound to concanavalin A-Sepharose and was eluted off by  $\alpha$ -methyl mannose. Inhibitory activity of the acidic isoferritins was detected at concentrations as low as  $10^{-17}$ - $10^{-19}$  M and iron saturation did not appear to be necessary for its action. These results implicate acidic isoferritins in the regulation of normal myelopoiesis and suggest a role for them in the progression of leukemia.

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