LACTOFERRIN, AN IRON-BINDING PROTEIN NI NEUTROPHILIC LEUKOCYTES*

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In 1939 a red protein was discovered in bovine milk by Sørensen and Sørensen (1). It has recently been isolated and its physicochemical features were described by several workers who investigated the bovine component (2, 3), as well as its human homologue (4–8). This protein has been given various names, such as lactoferrin (9), lactotransferrin (10), lactosiderophilin (10), or ekkrinosiderophilin (11). It shares with serum transferrin the property of reversibly binding two atoms of iron. However, the two proteins differ greatly in their antigenic properties (6, 7) and chemical composition (12–16).

On account of its absence in serum, lactoferrin has been regarded as a specific milk protein until 1963 when Biserte et al. (17) reported its occurrence in the sputum of bronchitis patients. Using immunological methods, it has also been possible to demonstrate the presence of lactoferrin in various other biological fluids, as tears, saliva, nasal and bronchial secretions, gastrointestinal fluid, hepatic bile, urine, seminal fluid, and cervical mucus (18). Taken together, these findings have created the impression that lactoferrin is chiefly an epithelial secretion product. Recent investigations on guinea pig tissues have however shown that the spleen and bone marrow are rich sources of lactoferrin (unpublished). Furthermore, immunohistochemical observations on certain human tissues, the uterine cervix for example, suggest that glandular epithelia are not the only sites of origin for lactoferrin in external secretions and that certain blood cells may also be implicated in the production of this protein (19).

It seemed warranted, therefore, to undertake a search for the occurrence of lactoferrin in circulating blood cells and to investigate whether lactoferrin was actually synthesized in hematopoietic tissues. To answer these questions, the following experiments were carried out on human and guinea pig material.

Materials and Methods

Collection of Material.—

a. Human leukocytes were separated from red cells by accelerating the sedimentation of the latter by means of dextran. 16 ml samples of venous blood were collected in siliconized

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tubes containing 4 ml of 6% dextran (mol wt, 200,000) and 1% ethylene-diamine tetraacetic acid dipotassium salt in saline. The tubes were left to stand in a slanting position for about 20 min until the red cells had sedimented. The supernatant plasma, rich in white cells, was removed by pipetting and centrifuged at 250 *g* for 5 min. Usually the pellet contained some erythrocytes. These were eliminated by resuspending the cells in hypotonic (0.25%) saline for 30 sec. In some cases two or three hypotonic shocks were necessary. Platelets (which failed to sediment at 250 *g*) were collected as a separate fraction by centrifuging the leukocyte-free plasma at 2500 *g* for 10 min.

b. Guinea pig leukocytes were harvested from peritoneal exudates. The technique was the same as that employed by Hirsch (20) for the collection of rabbit leukocytes. Adult albino guinea pigs were injected intraperitoneally with 50 ml of 0.1% glycogen (shellfish glycogen, Sigma Chemical Co., St. Louis, Mo.) in pyrogen-free saline. After 4 hr 100 ml of heparinized saline was introduced into the peritoneal cavity and the exudate drained by gravity. The total number of leukocytes recovered by this method was about 120×10^6 cells. More than 95% of these were neutrophilic leukocytes. Contaminating erythrocytes were discarded as described for human material.

Processing of Cells.—Cells were disrupted by freezing and thawing 6 times. In most experiments, the leukocytes were extracted by means of 0.01 M citric acid in the proportion of 1 ml for 15×10^6 cells. After centrifugation at 105,000 *g* for 2 hr at 4°C (Rotor 40, Spinco Division, Beckman Instruments, Inc., Palo Alto, Calif.), the supernatants were concentrated by ultrafiltration or freeze-drying. For a clear visualization of electrophoretic bands and immunological precipitin lines, the material corresponding to 10^9 cells had to be concentrated to 0.75 ml. In the particular case of platelets, the extract was concentrated in the proportion of 0.75 ml for 2×10^9 platelets.

Analytical Methods.—Electrophoresis was carried out on cellulose acetate in pH 8.6 HCl-barbiturate buffer, using a Beckman Microzone apparatus. The same technique was also adopted for immunoelectrophoresis. For this purpose, a narrow strip of cellulose acetate foil was moistened with antiserum and carefully deposited on the carrier sheet alongside the electrophoretic run. X-ray film, Curix R.P. (Agfa-Gevaert, Antwerp, Belgium), was employed for the radioautographies. ^{59}Fe of high specific activity, provided by CEN (Centre d'Energie Nucléaire, Mol, Belgium), was used in the form of ferric citrate. Exposure times ranged from 24 hr to 10 days, according to the specific activity of the metal.

The procedure employed for the isolation of human lactoferrin has been reported elsewhere (21). A detailed description on the chemical properties of guinea pig lactoferrin is in preparation. Antisera against each of the two proteins were obtained from rabbits which were injected intramuscularly with 1 mg of antigen every 2 wk.

Quantitative determinations of lactoferrin were carried out by radial immunodiffusion in agarose gel as described by Mancini et al. (22). The plates were stained with Coomassie blue which significantly increased the sensitivity of the method.

Histological Techniques.—Specimens from normal human bone marrow were obtained by sternal puncture. Smears on microscopic slides were dried and fixed by means of absolute methanol for 3 min, washed in two successive baths of buffered saline (pH 7.2), and incubated with a drop of fluorescein-labeled (23) antiserum. Before use, the antiserum was absorbed with normal human serum, erythrocytes (Rh + AB), and rabbit liver powder.

For tissue cultures, Eagle's medium was used without addition of serum. Protein hydrolysate from *Chlorella vulgaris* (batch 42, The Radiochemical Center, Amersham, Buckinghamshire, United Kingdom) was employed as a source of ^{14}C-labeled amino acids. 1 ml containing 0.16 mg amino acids, corresponding to 100 μc, was diluted to 6 ml with Eagle's medium which contained all the amino acids except those present in the radioactive hydrolysate. Penicillin and streptomycin were added to the medium to a concentration of respectively 200 units/ml and 0.1 mg/ml.

RESULTS

I. Identification of Lactoferrin in Leukocyte Extracts.—

1. Electrophoresis on cellulose acetate: When extracts from peritoneal leukocytes of guinea pigs were fractionated by cellulose acetate electrophoresis, two cathodic bands could be distinguished. The slower band had the same migration rate as pure lactoferrin and was visualized on X-ray films after the addition of ^{59}Fe to the sample (Fig. 1). Essentially similar results were obtained with extracts from human blood leukocytes.

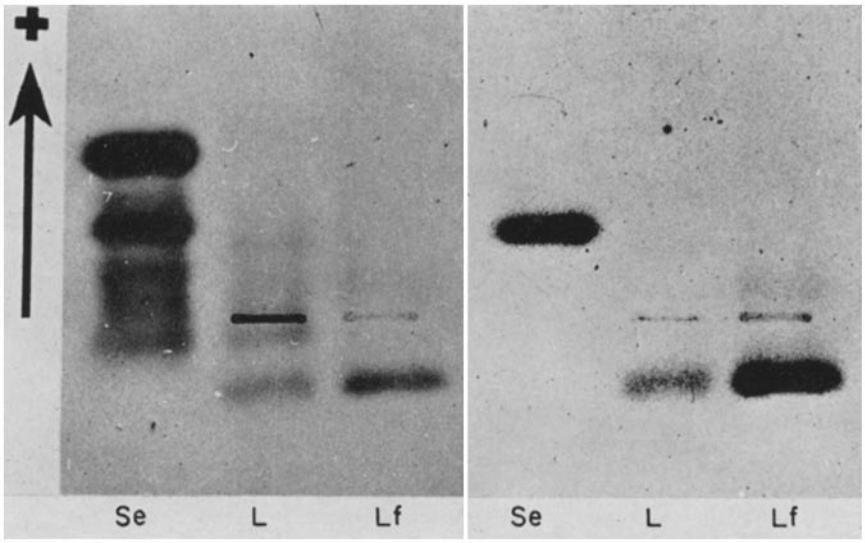

FIG. 1. Left: electrophoresis on cellulose acetate of guinea pig leukocyte extract (L), serum (Se), and lactoferrin (Lf) (Ponceau S staining). Right: the corresponding radioautography.

2. Immunoelectrophoresis: Specific antisera against human and guinea pig lactoferrin formed well-defined precipitin lines with the corresponding leukocyte extracts. After addition of ^{59}Fe to the samples, these precipitin lines could be revealed by radioautography (Fig. 2). Guinea pig lactoferrin migrated more slowly than its human homologue. Absorption of the antiserum with pure lactoferrin prevented the development of the precipitin line in the leukocyte extract (Fig. 3). The excess of antigen used for absorption reacted with the nonabsorbed antiserum diffusing from the opposite antiserum-containing strip, giving rise to a straight precipitin line. This fused with both ends of the precipitin arc from the leukocyte extract.

3. Ouchterlony plates (Fig. 4): Complete blending was observed between the

specific precipitin lines given by the human leukocyte extract and the sample of pure human lactoferrin, respectively.

II. Types of Leukocytes Containing Lactoferrin.—

To identify the cells involved, two methods were employed.

In the first method, leukocytes collected from 16 ml of human blood were fractionated by means of centrifugation in a concentrated solution of bovine albumin (24). This medium was prepared by dissolving 5 g of Cohn's Fraction

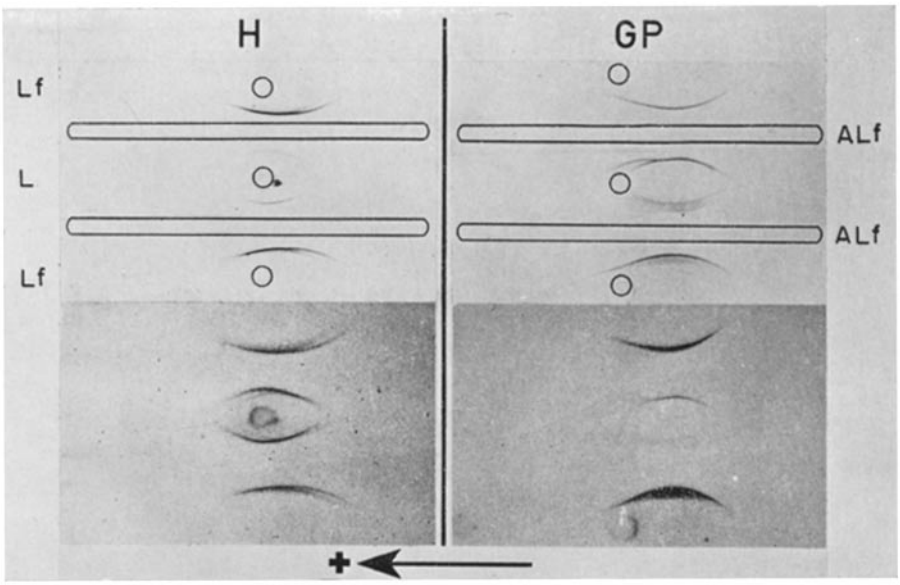

FIG. 2. Immunoelectrophoreses on cellulose acetate (top) and the corresponding radioautographs (bottom) of leukocyte extracts (L) developed by specific antiserum against lactoferrin (Alf). Left: human material (H). Right: guinea-pig material (GP). Lf, Lactoferrin.

V (Calbiochem, Los Angeles, Calif.) in 10 ml of water. After dialysis against saline, the density was adjusted to 1.067. The leukocyte sediment obtained as described under Methods was suspended in 4 ml of the albumin solution, and centrifuged at 2400 *g* for 36 min at 4°C. This yielded a compact pellet containing 77% polymorphonuclear cells and 23% mononuclear cells. The less dense monocytes were concentrated in a surface pellicle along with small numbers of lymphocytes (4%). The sedimented fraction, as well as the supernatant cells, after separation, were extracted separately and concentrated as described under Methods. As can be seen in Fig. 4, lactoferrin was detectable only in polymorphonuclear leukocytes. Not even trace amounts could be found in monocytes, nor in platelets which had been separated from the leukocyte-free plasma.

A more homogeneous preparation of neutrophilic leukocytes was obtained by collecting pleural exudate from a patient with acute pleuritis. This material contained 96% neutrophilic leukocytes. After enumeration, the cells were spun down, extracted, and concentrated in the usual manner. Lactoferrin was found not only in the cell sap but also in the unconcentrated fluid (Fig. 4).

Immunohistochemical studies confirmed the presence of lactoferrin in neu-

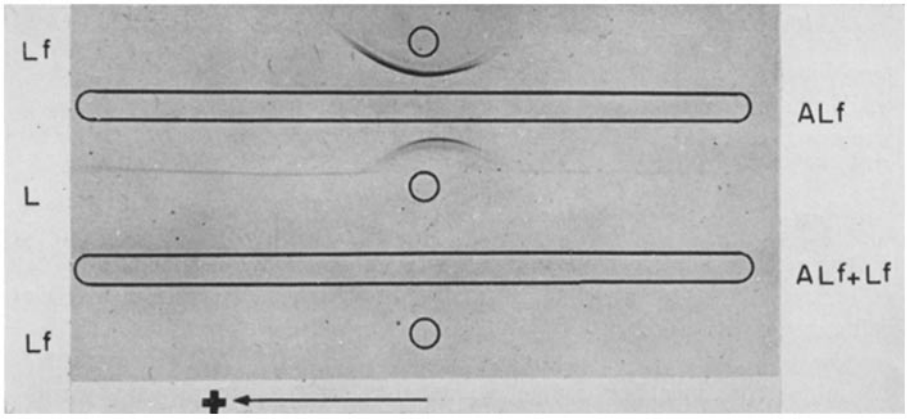

FIG. 3. Immunoelectrophoresis on cellulose acetate of human leukocyte extract (L) developed by specific antiserum against lactoferrin (Alf). The lower strip contained anti-lactoferrin absorbed in excess by means of pure lactoferrin (Lf).

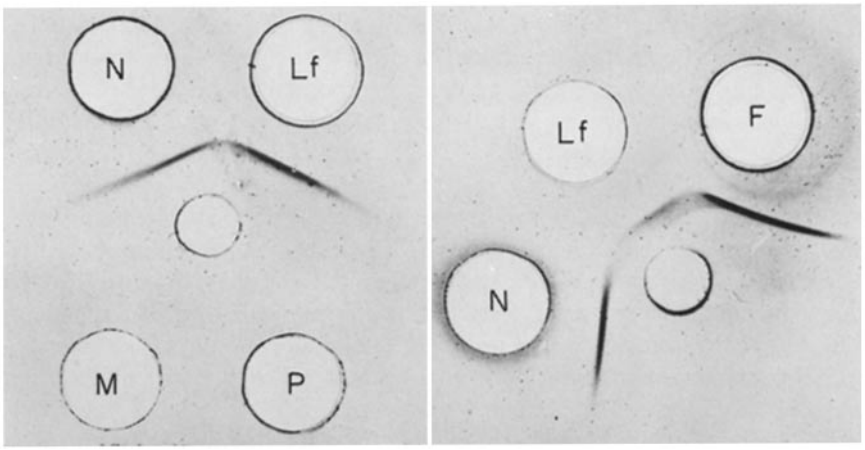

FIG. 4. Identification of lactoferrin (Lf) in cell extracts by means of immunodiffusion. Left: N, extract from sample rich in neutrophilic leukocytes. M, extract from sample rich in monocytes. P, extract from platelets. Right: N, extract from neutrophilic leukocytes of purulent pleural exudate. F, supernatant fluid from purulent pleural exudate.

trophilic leukocytes. In a smear of human bone marrow stained with fluorescein-labeled antiserum against lactoferrin, neutrophilic leukocytes displayed a bright cytoplasmic fluorescence (Fig. 7 and 8). The specificity of this fluorescence was demonstrated by the extinction of the reaction (Fig. 7) after absorption of the antiserum by means of purified antigen (2 mg of lactoferrin per ml of fluorescein-labeled antiserum). In the control smears, a few cells, identified as eosinophilic leukocytes, retained their fluorescence after such absorption, showing the nonspecific nature of their reaction with the antiserum (Fig. 7).

From the various pictures, it appeared that the lactoferrin content of neutrophilic cells increased with their advancing maturity. The brightest fluorescence was seen in metamyelocytes and in the mature forms. Faint staining was observed in what could presumably be considered to be promyelocytes (Fig. 8).

III. Extraction of Lactoferrin from Human Leukocytes.—

Owing to its relatively high isoelectric point (pI of human lactoferrin $\simeq$ 6.1) lactoferrin tends to be absorbed on acidic macromolecules such as nucleic acids or mucopolysaccharides (25). The recovery of lactoferrin from cell homogenates therefore requires extractants of low pH or high ionic strength. In the initial experiments, extraction with 0.01 M citric acid was routinely employed, but it was found that part of the lactoferrin was lost as a precipitate as soon as the pH was raised to the value required for electrophoretic or immunological analyses. Consequently, it was decided to test different solutions of increasing salt concentration for their suitability to the extraction of lactoferrin from leukocyte suspensions.

About 20 ml of human blood was employed for this study. After sedimentation of the red cells, the plasma containing 5000 neutrophilic leukocytes per mm^3 was divided into nine 1 ml portions. After centrifugation, each tube was carefully drained on filter paper and the pellets were resuspended in 0.1 ml of the various solutions to be tested: 0.9% sodium chloride; 0.01 M citric acid; 0.1 M phosphate buffer of pH 8; and the same buffer containing respectively 0.3, 0.6, 1.0, 1.3, 1.6 and 2.0 M sodium chloride. The samples were then frozen and thawed six times and left for 2 hr at 4°C with strong mixing every 15 min. The extracts were clarified by centrifugation at 6000 *g* for 2 hr at 4°C. The clear supernatants were tested for their lactoferrin content in radial immunodiffusion plates which had been prepared with agarose dissolved in the corresponding extractant solutions. The sample extracted with citric acid solution was assayed in a phosphate-buffered gel plate. Each plate included five standard dilutions of purified lactoferrin ranging from 50 μg/ml to 300 μg/ml. The results are set out in Table I and indicate that the most efficient extraction media were phosphate-buffered saline solutions containing at least 1 M sodium chloride.

To verify whether the lactoferrin set free by the extraction was quantitatively assessed by the immunodiffusion procedure, known amounts of purified human lactoferrin were incorporated into each sample prior to diffusion, and their recovery was tested (Table I). It was found that the most efficient extractants were also those that permitted full recovery of the added lactoferrin. With citric acid or normal saline as the extractant, several precipitin rings were observed, whereas single rings were formed by extracts obtained by means of buffered solutions of high ionic strength.

TABLE I

Yield from Extraction of Leukocyte Lactoferrin by Various Solvents

Extraction media	Lactoferrin extracted from 10^6 neutrophilic leukocytes	Recovery of standard added to the sample*
	(μg)	%
0.9% NaCl	0.35	—
0.01 M citric acid	0.97	57
0.1 M phosphate, pH 8.0	1.25	45
" " " 0.3 M NaCl	1.68	95
" " " 0.6 M "	2.24	93
" " " 1.0 M "	3.06	103
" " " 1.3 M "	2.85	105
" " " 1.6 M "	3.08	107
" " " 2.0 M "	2.96	109

* To each sample were added three different amounts of purified human lactoferrin (respectively 0.6, 0.4, and 0.2 μg) and the increments in precipitin ring size were compared to the ring sizes given by corresponding amounts of lactoferrin dissolved in the extractant.

IV. Biosynthesis of Lactoferrin by Hematopoietic Tissues.—

1. Incorporation of labeled amino acids: For this experiment five albino guinea pigs, 1 wk old, were used. The marrow was extruded from their tibial bones by injecting into the medullary cavity a few ml of Hanks' solution containing 40 IU of heparin per ml. A total of about 200 × 10^6 bone marrow cells were recovered from the five animals. After dispersion by gentle pipetting, the cells were washed with Hanks' solution and resuspended in 6 ml of culture medium to which were added 2 ml of the ^{14}C amino acid solution, as described under Methods. The preparation was then equally divided into two Erlenmeyer flasks, one of which was frozen without delay, while the other was incubated at 37°C on a moving tray (40 cycle/min). After 48 hr, 10 ml of 0.01 M citric acid was added to each of the flasks and the mixtures were frozen and thawed six times. Insoluble material was removed by centrifugation at 105,000 *g* for 2 hr at 4°C. The supernatants were concentrated to 0.2 ml by ultrafiltration and dialyzed for 48 hr against 1 liter of saline, which was renewed three times.

Immunoelectrophoreses of the concentrated culture media and their controls were developed by means of specific antiserum against guinea pig lactoferrin. Radioautographies were exposed for 3, 14, and 30 days, respectively. The bone marrow culture incubated at 37°C gave rise to a strongly radioactive precipitin line which could already be detected after a 3 days' exposure (Fig. 5). A very faint line similar in shape and position was visible on the films that had been exposed for 30 days to the immunoelectrophoresis of the control sample.

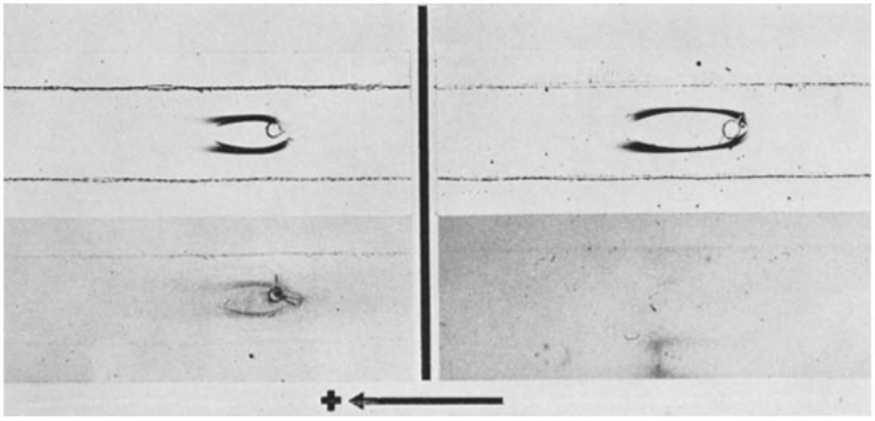

FIG. 5. Immunoelectrophoreses in agarose (top) and corresponding radioautographs (bottom) of extracts from guinea pig bone marrow incubated at 37°C (left) or stored at −5°C (right) in the presence of ^{14}C-labeled amino acids. Antiserum specifically reacted with lactoferrin. Radioautographies were exposed for 3 days.

To exclude the possibility that the radioactivity associated with lactoferrin from bone marrow cultures might have been due to adsorption of the labeled amino acids to the protein rather than to their incorporation into the molecule, the following experiment was carried out. Radioactive culture extract was added to unlabeled carrier consisting of 10 ml of guinea pig milk whey, and this mixture was fractionated by chromatography on DEAE-cellulose (2 g, 0.7 mEq/g), using stepwise elution with pH 7.6 phosphate buffer of increasing ionic strength. Prior to application to the column, the sample had been equilibrated with the starting buffer (0.01 M phosphate). The concentrated eluates were analyzed by electrophoresis on cellulose acetate (Fig. 6). The major part of the lactoferrin emerged from the column with the breakthrough fraction, which also contained material migrating as immunoglobulins. Both the zone corresponding to the latter proteins and that corresponding to lactoferrin appeared clearly on films exposed for 10 days. None of the fractions subsequently eluted from the column displayed any radioactivity detectable after the same exposure time.

2. Net synthesis of lactoferrin by the bone marrow: The preceding data indicated that lactoferrin was very actively synthesized by bone marrow tissue. It was therefore attempted to obtain direct proof of net synthesis of this pro-

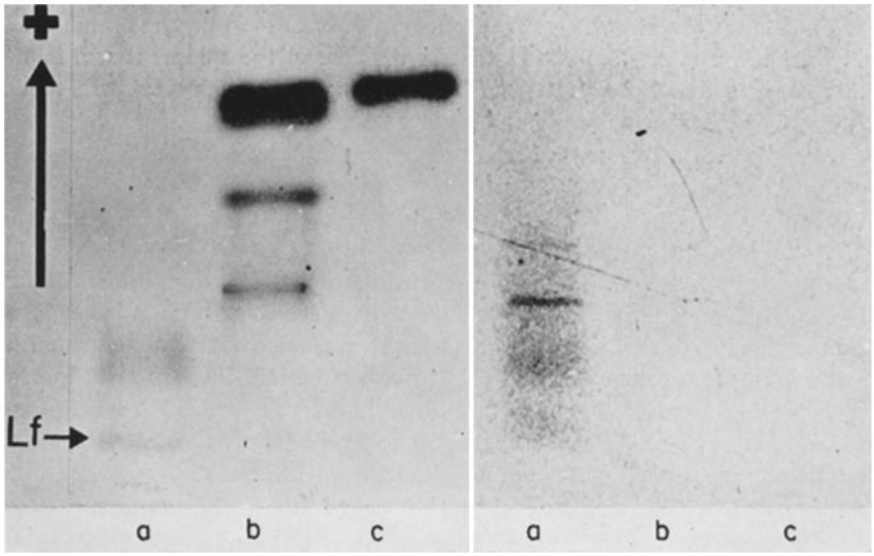

FIG. 6. Electrophoresis on cellulose acetate (left) of fractions of DEAE-cellulose chromatography from a mixture of guinea pig milk whey and extract of bone marrow cultured in the presence of ^{14}C-labeled amino acids. The corresponding radioautography (right) was exposed for 10 days.

TABLE II

Production Rate of Lactoferrin by Cultures of Hematopoietic Tissues from Guinea Pigs

Experiments	Tissue (pooled)	Total amount of lactoferrin recovered from culture	
		Incubated at 37°C	Frozen at −5°C
		μg	μg
1	Bone marrow (n*, 2)	220	140
2	Bone marrow (n, 5)	1248	570
3	Spleen (n, 5)	405	185

* n, Number of animals used.

tein by marrow cultures because such evidence would seem to be superior to that based solely on the incorporation of labeled precursor molecules.

In the first experiment, tibial bone marrow collected from two 3-month old guinea pigs was processed as described for the isotopic incorporation assay, except that no radioactive precursors were added. After extraction and concen-

tration to 0.5 ml, the culture material and its cold-inhibited control were assayed in immunodiffusion plates for the quantitation of their lactoferrin contents. It was found (Table II) that the culture which had been incubated at 37°C contained about 1.5 times as much lactoferrin as the control.

Similar experiments were carried out on the bone marrow and spleen from five animals 1 month-old except that in the case of the spleens it was found necessary to dissociate the tissue by squeezing it through fine-mesh metal gauze. The marrow and spleen extracts were concentrated, respectively, to 3 ml and 1 ml. Lactoferrin was found to present in far greater amounts in the cultures incubated at +37°C than in the controls kept at −5°C.

DISCUSSION

The results presented here indicate that leukocytes contain a protein which is identified as lactoferrin on the basis of its electrophoretic, antigenic, and iron-combining properties. No confusion was possible with serum transferrin, an iron-binding protein reported (26) to be synthesized by peritoneal macrophages from the rat. Not only have transferrin and lactoferrin never been found to cross-react in immunological assays (6, 7), but in our experiments the additional precaution was taken to absorb the anti-lactoferrin antisera with serum from the species which had furnished the antigen, and the completeness of this absorption was verified by the absence of precipitation in gel with the corresponding serum. Also, the electrophoretic mobilities of the proteins identified as human and guinea pig lactoferrin exactly corresponded to those of the corresponding purified milk lactoferrin samples.

Both in the guinea pig and the human, the white blood cells containing lactoferrin were identified as polymorphonuclear leukocytes. The glycogen-induced peritoneal exudates used in this study were harvested 4 hr after injection of the irritant, i.e., at a time when the population of cells in the exudate consisted of more than 95% of polymorphonuclear leukocytes, as verified in our samples. Lactoferrin was recovered in large amounts in the extract from these cells (Fig. 1). It was also found in a granulocyte-rich fraction from human blood as well as in pus cells from a human pleural exudate, whereas none could be detected in a 96% pure suspension of human blood monocytes, nor in human platelets.

In human material, the polymorphonuclear leukocytes containing lactoferrin clearly belonged to the neutrophilic cell line. This conclusion is derived both from immunohistochemical evidence (Figs. 5, 6) and from the fact that neutrophils were the only cell type found in the purulent exudate shown to be rich in lactoferrin. The findings reported here do not exclude the occurrence of lactoferrin in eosinophilic leukocytes, owing to the nonspecific fluorescence displayed by these cells upon exposure to fluorescein-labeled antiserum. However, the low amounts of lactoferrin found in peripheral leukocytes of patients

with pronounced eosinophilia (unpublished) seem to rule out the eosinophil as a major source of this protein.

The immunohistochemical data show that lactoferrin first appears at the stage of the promyelocyte and that its concentration in the cytoplasm progressively increases until the stage of the fully mature neutrophilic leukocyte. It remains to be demonstrated whether the protein is confined to granules or distributed throughout the cell sap.

Although red blood cells fail to react with fluorescein-labeled antilactoferrin, the possibility of lactoferrin being present in erythroblasts cannot be excluded at present.

The quantitative extraction of lactoferrin from human white blood cells is best achieved by means of phosphate-buffered solutions of high ionic strength. With this technique, the lactoferrin content of human neutrophilic leukocytes was found to be about 3 μg of lactoferrin per 10^6 cells. Lactoferrin was also one of the major soluble proteins recovered in the extracts from guinea pig peritoneal leukocytes, as can be seen on the electrophoretic pattern illustrated in Fig. 1.

The tissue culture experiments demonstrated that hematopoietic tissues were able to synthesize lactoferrin. In previous publications it has been reported that lactoferrin is present in the epithelial cells of glandular acini from human bronchi (23) and submaxillary glands (21), and that this protein is actively synthesized by tissue cultures from human rectal mucosa and uterine cervix (19).

There appears to exist a remarkable parallel between the histological distribution of lactoferrin and that of lysozyme. It has been reported that the lytic activity of lysozyme is raised and extended to several kinds of otherwise insensitive bacteria in the presence of chelating agents (27). Since lactoferrin is a powerful chelator of iron (2, 6, 7) as well as of copper (8), it is tempting to speculate that this protein may be a valuable adjunct of lysozyme in the defense against bacterial invasion. Moreover, lactoferrin is by itself endowed with bacteriostatic properties which it owes to its iron-chelating capacity (21, 23, 28). It would also be interesting to clarify the possible relationship of lactoferrin with "phagocytin", the antimicrobial factor from polymorphonuclear leukocytes described by Hirsch (20). The role here suggested for leukocytic lactoferrin is based on the assumption that this protein is not already saturated with iron before it leaves the cells. Although unproven, this is very likely, since lactoferrin present in human milk is known to be highly unsaturated (29).

Yet another speculation may be ventured as to the impact of leukocytic lactoferrin upon the metabolism of iron (30). The complex between iron and serum transferrin is known to be largely dissociated upon acidification below pH 5 (31). The lactoferrin-iron complex, in contrast, remains stable until pH 3 (2, 6, 7). Furthermore, the affinity of lactoferrin for iron appears to exceed that

of serum transferrin even in the physiological pH range (32). Considering that lactoferrin occurs in the fluid as well as in the cells of inflammatory exudates (Fig. 4), and in view of the low pH known to prevail in inflamed tissues (33), it would be surprising if the serum transferrin in the interstitial fluid bathing these sites were allowed to return to the circulation with the full load of iron it possessed upon leaving the vessels. Such an explanation would account for the low serum iron levels characteristic of inflammatory conditions.

SUMMARY

Lactoferrin, an iron-binding protein previously shown to occur in many external secretions, is identified as one of the major proteins present in human and guinea pig neutrophilic polymorphonuclear leukocytes.

The identification of this protein in leukocyte extracts was based upon a comparison of its electrophoretic, antigenic, and iron-combining properties with the corresponding properties of the same protein isolated from human and guinea pig milk. Immunochemical quantitations showed that lactoferrin occurs in human neutrophilic leukocytes at the concentration of 3 μg per 10^6 cells. Tissue cultures from guinea pig bone marrow and spleen actively synthesized the protein, as shown both by net production of lactoferrin and incorporation of labeled amino acids into the protein. Immunohistochemical data indicate that lactoferrin first appears in myeloid cells at the stage of the promyelocyte.

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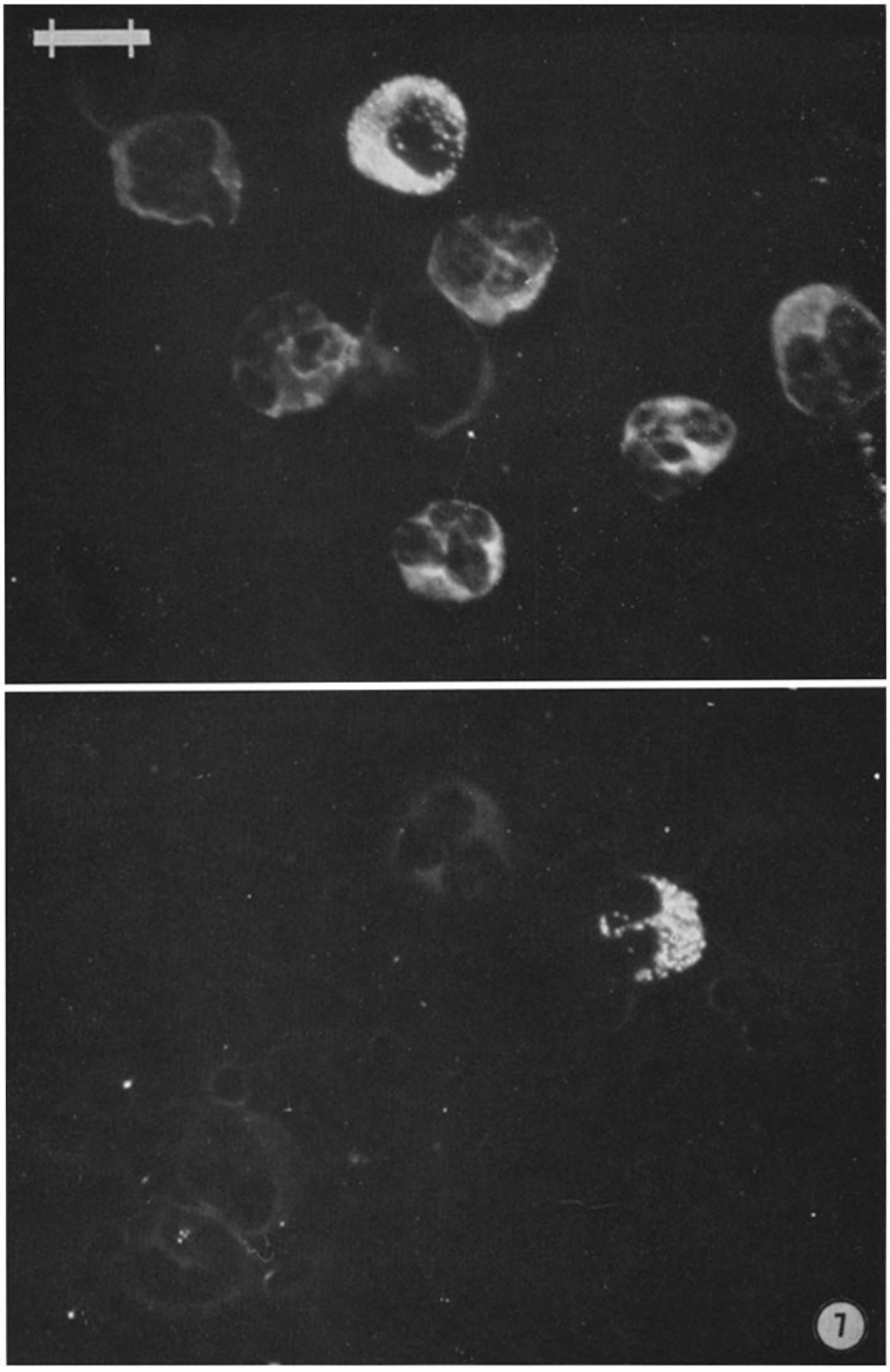

FIG. 7. Top: smears of human bone marrow stained with fluorescein-labeled antiserum against lactoferrin. Bottom: same, after absorption of the antiserum with pure lactoferrin. Index, 10 microns.

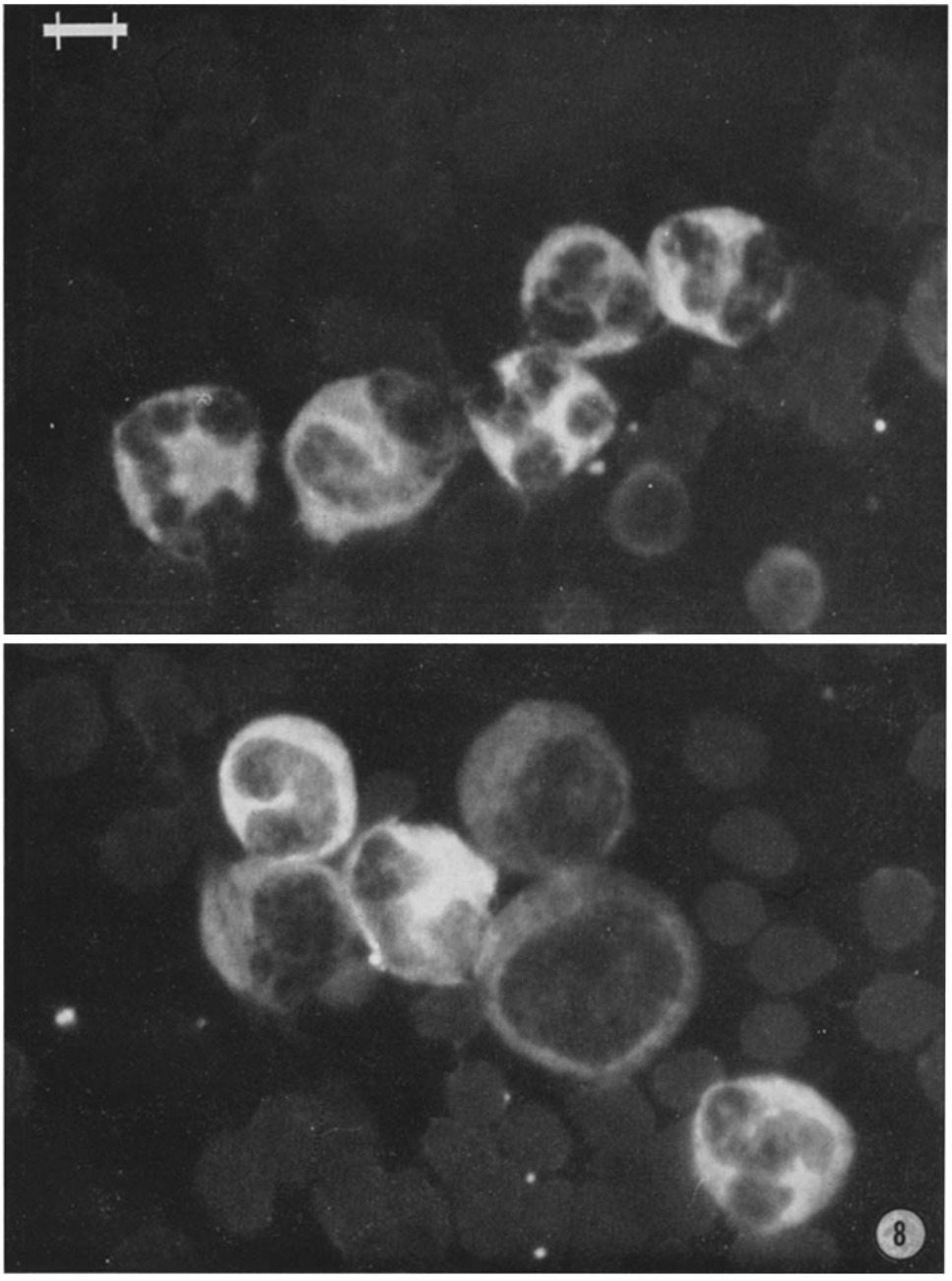

FIG. 8. Smears of human bone marrow stained with fluorescein-labeled antiserum against lactoferrin. Index, 5 microns.