THE INVOLVEMENT OF LACTOFERRIN IN THE HYPOSIDEREMIA OF ACUTE INFLAMMATION*

BY JACQUES L. VAN SNICK, PIERRE L. MASSON, AND JOSEPH F. HEREMANS

(From the Department of Experimental Medicine, University of Louvain, B-1200 Bruxelles, Belgium)

The concentration of iron in the plasma decreases during inflammation (1); it will, for instance, drop very sharply within 12 h after the injection of bacterial endotoxin (2) or the production of a turpentine abscess (3). This hyposideremia corresponds to the accumulation of iron, in the form of ferritin, in inflamed tissues and in the reticuloendothelial system (RES) (1, 4). Blockade of the latter prevents the development of hyposideremia (3).

On the other hand, neutrophilic leukocytes are required for the development of inflammatory hyposideremia, as shown by experiments with leukopenic animals (2, 5, 6). Kampschmidt and Upchurch (6), as well as Pekarek and Beisel (7) have attempted to characterize a leukocyte factor responsible for the phenomenon. Our own group has proposed (8) to explain the hyposideremia of inflammation, as being mediated by an iron-binding protein, lactoferrin, whose presence in the specific granules of neutrophilic leukocytes we have described (9, 10).

Lactoferrin is a glycoprotein sharing several biochemical properties (11–13) with transferrin. These two proteins have the same molecular weight (14) and both will reversibly bind two ferric ions with the incorporation of two molecules of bicarbonate (15). By their amino acid composition (14) they are clearly related, although they fail to show any immunological cross-reaction unless denatured (16). Lactoferrin further differs from transferrin by its affinity for iron being largely retained at pH values below 4.0 (11–13, 17), whereas under such conditions transferrin completely releases its iron. Yet another difference resides in the histological distribution of the two proteins. Whereas transferrin is a plasma protein synthesized mainly by the liver (18), lactoferrin occurs in the plasma only in trace amounts, but abounds in external secretions (8, 19) and neutrophils (9).

The present report will deal with experiments demonstrating that lactoferrin plays a determinant role in inflammatory hyposideremia. It is proposed that iron-free lactoferrin (apolactoferrin), when released from neutrophils, removes the iron from transferrin and, together with its load of iron, is selectively taken up by the RES.

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Materials and Methods

Reagents. Zymosan was obtained from Koch-Light Laboratories, Ltd., Colnbrook, England; endotoxin (*Escherichia coli* 0.55:85 lipopolysaccharide B) from Difco Laboratories, Inc., Detroit, Mich.; and human transferrin devoid of iron (apotransferrin) from Behring-Institut, Marburg, W. Germany.

Human lactoferrin was isolated from milk by chromatography on carboxymethyl-Sephadex (14). Some batches were prepared after saturation of the protein with iron (Fe-lactoferrin). One sample was isolated in a form poor in iron (13% saturation calculated from the absorbance at 460 nm). The latter sample will be referred to as native apolactoferrin, to distinguish it from the protein artificially deprived of iron by dialysis against 0.1 M citric acid (14).

Goat antiserum against the proteins extracted from rat heterophil leukocytes was obtained by repeated injections of material from 10^8 leukocytes. After absorption with rat plasma, this antiserum developed several precipitin lines with the immunizing antigen. The line due to lactoferrin was identified by autoradiography as described in the Results (Fig. 6).

Rabbit antiserum specific for rat lactoferrin was obtained as follows. Rat lactoferrin was isolated from extracts of peritoneal exudates (see below) by small-scale preparative electrophoresis in agarose gel at pH 8.6. The zone of gel containing lactoferrin was disrupted in 1 M saline by repeated freezing and thawing, and samples of this material containing 100–200 μg of protein were injected at 2-wk intervals into the foot pads of a rabbit. Freund's complete adjuvant was added to the first two doses of antigen. The antiserum was collected 1 wk after the fourth injection. After absorption with rat plasma it was found to be specific for lactoferrin, when tested with extracts of rat heterophil leukocytes (Fig. 6).

A goat antiserum directed against succinylated human lactoferrin was obtained by intradermally injecting, every 2 wk, 100 μg of succinylated Fe-lactoferrin suspended in 1 ml of complete Freund's adjuvant. Succinylation was carried out by dissolving 50 mg of Fe-lactoferrin in 3 ml of 0.2 M NaCl and 0.1 M $NaHCO_3$ and treating the protein at room temperature with 25 mg of succinic anhydride, added slowly over 1 h. The pH was maintained at 8.5 by addition of 0.1 M NaOH.

Specific antisera against human or rabbit lactoferrin were prepared in goats, as previously described (9, 10). Goat antisera against human albumin and transferrin, as well as reference standards were provided by our department of clinical chemistry.

Immunofluorescence was carried out with goat antiserum to human lactoferrin. The immunoglobulins, prepared by successive precipitation with rivanol and ammonium sulfate, were labeled with fluorescein isothiocyanate at a ratio of 10 μg of fluorochrome/1 mg of protein.

Animals. All the rats used for the present work were 3-mo old Wistar females weighing about 200 g. Intravenous injections were done via the tail vein. For repeated blood sampling from the same animal, the tip of the tail was bled into heparinized hematocrit tubes. Exsanguination was performed by puncturing the aorta.

Processing of Leukocytes. Human leukocytes from venous blood were separated from red cells by accelerating the sedimentation by means of dextran, as previously described (9). Rabbit and rat heterophil leukocytes were harvested from peritoneal exudates, following the procedure described by Hirsch (20). Cells were disrupted by freezing and thawing six times, and extracted by means of 1 M saline (9).

Analytical Methods. Immunoassays were performed by radial diffusion (21) or nephelometry in the Technicon Auto Analyzer (Technicon Instruments Corp., Tarrytown, N. Y.) (22, 23). Reference standards for the determination of rat lactoferrin were set up using an extract from rat leukocytes. The lactoferrin content of this material was estimated by densitometry scanning (Analytrol, Beckman Instruments, Inc., Fullerton, Calif.) of electrophoreses on cellulose acetate, using human lactoferrin as a standard. The electrophoretic band corresponding to lactoferrin was identified by autoradiography, as described in the Results. Lysozyme activity was estimated by radial diffusion in agarose gel containing *Micrococcus lysodeikticus* (24). Alkaline phosphatase was determined enzymatically with *p*-nitrophenyl phosphate as the substrate (Merckotest; E. Merck AG, Darmstadt, W. Germany). X-ray films routinely used in clinical departments were employed for the autoradiographies. ^{59}Fe of high specific activity was used in the form of ferric citrate.

Blockade of the RES. The RES of rats was blocked by intravenous injections of carbon particles,

following the procedure described by Biozzi et al. (25). Carbon particles (C11-1431A India ink from Gunther Wagner, Hannover, W. Germany), were centrifuged at 5,000 rpm for 15 min to eliminate the largest fragments (more than 25 nm), and were suspended in saline containing 2% gelatin in a proportion of 30 mg of carbon/ml. This preparation was stored in the cold, and was melted at 37°C before use. In the initial experiments, blockade of the RES was obtained by three injections of 30 mg of the colloid at 8-h intervals. It later appeared that a single injection was sufficient.

In order to detect possible specific interference of carbon particles with immunoassays, some experiments of RES blockade were also performed by single intravenous injections of aggregated human albumin. The latter protein, at a concentration of 30 mg/ml in saline, was aggregated at pH 10.0 by heating at 70°C for 20 min and then at 79°C for another 15 min, under permanent shaking at a rate of 100 times/min (26). Aggregation was monitored by the increase of absorbance at 520 min. After adjusting the pH at 7.5, the colloid was stored at 4°C until use.

Results

In Vitro Experiments

Release of Apolactoferrin by Neutrophil Leukocytes. The release of lactoferrin by rabbit heterophils was studied on cells collected from peritoneal exudates. On the average, about 0.5×10^9 cells consisting for more than 90% of heterophils were obtained from one animal. After brief centrifugation, the cells were suspended in saline enriched with 5% glucose and separated into two fractions. The final cell number ranged from 10 to 40×10^6/ml. One part of the suspension was incubated at 37°C with 100 mg of zymosan, the rest being used as a control. Both samples were centrifuged after 45 min. The cell pellets were discarded and determinations of lactoferrin, lysozyme, and alkaline phosphatase were carried out on the supernates. Lactoferrin was measured by radial immunodiffusion, and its concentration was expressed in micrograms per milliliter, with extracts of rabbit neutrophils as standards, assuming the lactoferrin content of rabbit heterophils to be similar to that of human neutrophils, i.e., 3.4 μg of lactoferrin/10^6 cells. The induction of phagocytic activity by zymosan caused the simultaneous release of lactoferrin, lysozyme, and alkaline phosphatase, as well as a slight decrease in the pH of the medium (Table I).

TABLE I

Changes in the Concentration of Lactoferrin, Enzymes, and Hydrogen Ion in the Medium Upon Incubation of 0.5×10^9 Rabbit Heterophil Leukocytes with Zymosan*

Lactoferrin (μg/ml)		Lysozyme (μg/ml)		Alkaline phosphatase (mU/ml)		pH	
Phagocytosis	Control	Phagocytosis	Control	Phagocytosis	Control	Phagocytosis	Control
246	36	102	44	—	—	6.9	7.3
102	23	81	32	—	—	7.8	8.0
38	21	61	30	130	60	6.8	6.9

* Extracts of neutrophils were used as standards, with the assumption that 10^6 neutrophils contained 3.4 μg of lactoferrin.

An experiment with human neutrophils was also carried out. The cells harvested from 100 ml of venous blood were exposed to *Staphylococcus albus* which had been killed by heating. The leukocytes were resuspended in 1 ml of plasma. One half of each sample was mixed with 20 μl of the pellet of bacteria, the rest serving as a control. After 1 h of incubation at 37°C, lactoferrin was found to have reached a concentration of 100 μg/ml in the fluid phase of the sample exposed to bacteria, against only 17 μg/ml in the control.

To verify whether the lactoferrin discharged from neutrophils was in the iron-free form or fully saturated with metal, a leukocyte extract was prepared to which [^{59}Fe]citrate was added. Leukocytes were isolated from 100 ml of human blood and lactoferrin was extracted as described in the Materials and Methods.

Special care was taken to avoid contamination with iron from glassware or reagents. In order to eliminate the traces of metal ordinarily present in saline (0.5 mg iron/g of NaCl), the solutions used for washing the cells and extracting the proteins were passed over a column of apolactoferrin coupled to Sepharose (unpublished observations). To remove plasma proteins, the pellet of leukocytes (about 100 μl) was washed three times with 0.15 M saline and extracted with 1 ml of 1 M NaCl, after which 400 nmol of ^{59}Fe in 0.5 M citrate was added to the sample.

The proteins were separated from the Fe-citrate complex by filtration on Sephadex G25 which had been equilibrated with 1 M NaCl containing 0.05 M citrate, a concentration of chelator known to prevent the formation of ferric citrate polymers (27). Fractions of 0.5 ml were collected, counted for radioactivity, and assayed for lactoferrin by nephelometry. All the iron eluted with the proteins was bound to lactoferrin, since the latter was the only radioactive protein detectable by electrophoresis on cellulose acetate of similar extracts. In particular, the absence of transferrin in the sample was verified by an immunologic precipitin test.

From the Fe to lactoferrin ratio in the various fractions eluted from Sephadex G25, it was evident that the lactoferrin extracted from the leukocytes had fixed ^{59}Fe up to 92% (SD, 9.3%) of its iron-binding capacity.

Exchange of Iron between Transferrin and Lactoferrin. Apolactoferrin and half-saturated ^{59}Fe-labeled transferrin were mixed in equal proportions (5 mg/ml for each). After 1-h incubation at 37°C, in various media, the lactoferrin and transferrin from 1-ml portions of the mixture were separately removed by means of short immunosorbent columns made with the appropriate antisera and equilibrated with the same medium as that used for the incubation. The recovery of each protein, after elution with 3 M ammonium thiocyanate, was monitored by the absorbance at 280 nm, and the radioactivity in each eluate was counted.

The results (Table II) showed that in the absence of citrate, lactoferrin was unable to remove any iron from transferrin at pH 7.3 (0.01 M phosphate), whereas 50% of the transferrin iron was passed on to lactoferrin at pH 6.65 (0.01 M phosphate), and 75% at pH 5.6 (0.1 M acetate buffer). In the presence of 10 mM citrate, these proportions became approximately 50% (pH 7.3), 50% (pH 6.6), and 95% (pH 5.6), respectively, indicating a facilitating effect of the chelator during the iron exchange.

In an attempt to reproduce physiopathological conditions, experiments were

TABLE II
Uptake of ^{59}Fe by Lactoferrin from 50% Saturated Transferrin

	pH 7.3	pH 6.6	pH 5.6
No citrate	0–4*	57–58	73–79
Citrate (10 mM)	47–53	39–54	94–95
Pus ultrafiltrate		57–58	

* Percent of the iron originally bound to transferrin.

also performed with lactoferrin and transferrin dissolved in the ultrafiltrate from a purulent pleural exudate. The pH of this medium was 6.6. The amount of iron removed by lactoferrin was found to be 57–58% of the metal originally present on transferrin.

The latter experiment was repeated with human serum at pH 7.4 containing 2.9 mg/ml of 35% saturated transferrin. The sample (4 ml) was labeled with a trace amount of ^{59}Fe (100 μl of ^{59}Fe in 0.025 M citrate). Apolactoferrin was dissolved in the serum to a final concentration of 3 mg/ml. After a 1-h or 4-h incubation at 37°C, lactoferrin and transferrin were extracted by immunosorbents. In four different experiments it was found that lactoferrin had acquired 2.5–10% of the iron originally present on transferrin.

Uptake of Lactoferrin by Mononuclear Blood Cells. Human Fe-lactoferrin was added, to a final concentration of 5 mg/ml, to human leukocytes isolated from 10 ml of blood and suspended either in 4 ml of plasma or 0.15 M saline. In the first experiment, in order to prevent pinocytosis of the protein, the leukocytes were exposed to lactoferrin in the cold (4°C) and were then washed three times with cold saline; the cell pellet was resuspended in cold fluorescein-labeled goat antiserum to lactoferrin, and again incubated for 1 h at 4°C. After washing with cold isotonic saline, a drop of the cell suspension was examined under the UV microscope. Thin crescents of fluorescence were seen surrounding mononuclear cells (Fig. 1). This typical membrane fluorescence was not visible on cells that had not been exposed to lactoferrin. Nonspecific adsorption by leukocytes of fluorescent antibodies was also ruled out by the absence of fluorescence on cells incubated with fluorescein-labeled immunoglobulins from a goat immunized against albumin. When examined by phase contrast, the lactoferrin-binding cells were identified as monocytes on the basis of their size and the shape of their nuclei (Fig. 1).

In a second experiment, the leukocytes were exposed to Fe-lactoferrin at 37°C for 1 h. After three washings the cells were spread on microscope slides, dried, and fixed for 3 min in absolute methanol. The smear, washed with 0.02 M Sørensen phosphate buffer, pH 7.2, was then stained with fluorescein-labeled antiserum to lactoferrin. Patches of fluorescence were visible in the cytoplasm of two types of cells, some clearly neutrophils, and the others having the morphology of monocytes (Fig. 1). On the control smear prepared with leukocytes not exposed to lactoferrin, only neutrophils were fluorescent. The specificity of the fluorescence observed in these two experiments was attested by the lack of

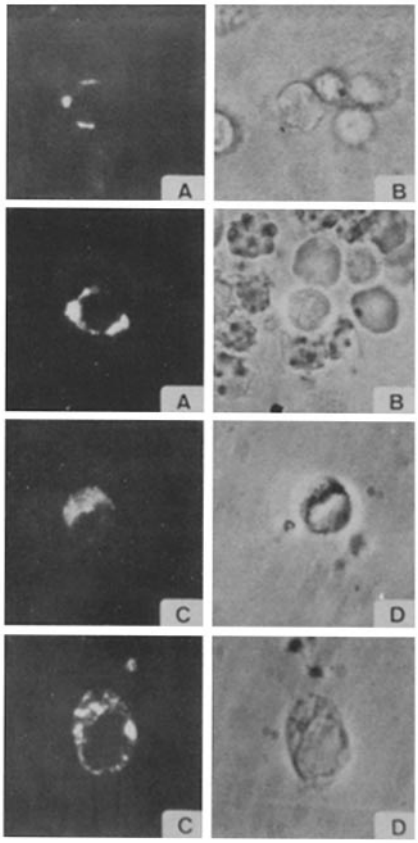

FIG. 1. Human blood monocytes having fixed (A and B) or ingested (C and D) human lactoferrin (revealed by fluorescein-labeled goat antiserum to human lactoferrin; B and D are phase-contrast photographs of A and C, respectively). A and B are living cells from buffy coats suspended in saline after exposure to a 0.3% solution of lactoferrin in serum for 1 h at 4°C, followed by washing with saline, and vital surface staining at 4°C with fluoresceinated antiserum. C and D are dried, fixed, fluoresceinated antiserum-stained cells from buffy coats that had been suspended in a 0.3% solution of lactoferrin in serum for 1 h at 37°C.

staining when the smears were exposed to the same antilactoferrin antiserum previously absorbed on a column of lactoferrin coupled to Sepharose.

In Vivo Experiments

Hyposideremia Induced by Exogenous Apolactoferrin. The intravenous injection into rats of 15 mg of human native apolactoferrin dissolved in 1 ml of saline caused on the average a 46% decrease in the plasma iron level measured 4 h after the injection (Table III). Human Fe-lactoferrin or human serum albumin, at the same dose did not cause any change, whereas human apotransferrin induced an increase of about 32% ($0.1 < P < 0.2$).

When graded amounts (5, 10, or 15 mg) of apolactoferrin were injected, the effect on the iron level was related to the dose of the protein (Fig. 2). For doses of 5 or 10 mg the plasma iron appeared to reach its minimum 2 h after the injection.

TABLE III

Plasma Iron Levels (μg/100 ml) of Rats 4 h after 15-mg Injections of Protein

	Apo-lactoferrin	Fe-lactoferrin	Apo-transferrin	Albumin	Nil
Mean	133.3	267	323	259.5	244
SD	38.7	44.5	40	32	23.4
Range	74-210	210-302	273-378	220-304	188-273
n	9	3	4	4	10

For 15 mg of apolactoferrin the hyposideremia continued unabated for at least 4 h. With the latter dose, but not with the smaller doses, significant amounts of lactoferrin were still detectable in the plasma 2 h after the injection. This suggested that what really mattered for the removal of iron from the circulation was not so much the amount of apolactoferrin injected or remaining in the circulation, as the quantity of the protein that had been cleared from the blood within the period of observation. This assumption was tested by determining the lactoferrin content of the plasma at different time intervals and for different doses of apolactoferrin injected. The plotting of these data (Fig. 3) brought out a strikingly linear correlation between the amounts of iron and lactoferrin cleared from the plasma. From this diagram, it appeared that the removal of 0.37 μg of iron corresponded to the clearance of 1 mg of lactoferrin. Hence lactoferrin, which is able to bind 1.4 μg of iron/mg, was eliminated at a saturation level corresponding to about 40% of its iron-transporting capacity, taking into account that the native apolactoferrin used in these experiments was saturated at 13% of its capacity when injected (see Materials and Methods).

Clearance of Exogenous Lactoferrin. Various human proteins (15 mg in 1 ml of saline) were injected intravenously into rats and their concentration was determined by nephelometric immunoassay in blood samples collected at regular intervals. Differences between the rates of elimination of these proteins were particularly marked in the first part of the clearance curves. Over the first 90 min these curves were simple exponentials (Fig. 4) from which an "initial half-life" could be calculated. The initial half-life of Fe-lactoferrin (Table IV) was particularly short (33 min; SD, 1.6 min), compared to that of Fe-transferrin (243 min; SD, 78 min) or albumin (165 min; SD, 34 min). When the sample of lactoferrin injected was nearly devoid of iron (native apolactoferrin), its initial half-life was significantly increased (53 min; SD, 3.8 min). This dependance of the rate of clearance on the degree of saturation with iron was confirmed by the results obtained with half-saturated lactoferrin whose half-life (44 min; SD, 3 min) was found to be intermediate between that of Fe-lactoferrin ($P < 0.01$) and that of native apolactoferrin ($P \simeq 0.05$). Succinylation of Fe-lactoferrin markedly prolonged its initial half-life (103 min; SD, 13.4 min), although its iron content (95%) remained unaltered by this treatment.

When the RES of the animals was blocked by India ink or aggregated albumin, the half-life of Fe-lactoferrin respectively increased about threefold (90 min; SD, 25 min) and fourfold (110 min; SD, 26 min).

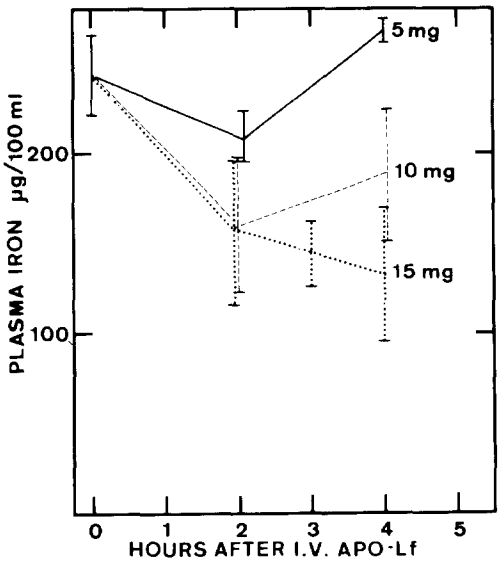

FIG. 2. Decrease of serum iron in rats after the intravenous injection of various amounts (5, 10, or 15 mg) of human apolactoferrin in saline. Vertical bars indicate the ±1 SD range. From 3 to 10 rats were used for each determination.

Uptake of Endogenous Fe-Lactoferrin by the RES

IDENTIFICATION OF LACTOFERRIN FROM HETEROPHILIC LEUKOCYTES OF THE RAT. When extracts from peritoneal leukocytes of rats were fractionated by cellulose acetate electrophoresis at pH 8.5, two very closely spaced and poorly resolved cathodal bands of equal intensity could be distinguished. Both bands showed up on X-ray films after addition of ^{59}Fe to the sample (Fig. 5).

The goat antiserum against extracts from rat leukocytes developed several precipitin lines with this material. Among these antigens, the one that migrated towards the cathode was capable of binding ^{59}Fe, as indicated by a autoradiography from the immunoelectrophoresis (Fig. 6). An antiserum prepared by injecting this cathodal protein into a rabbit (see Materials and Methods) formed a single precipitin line with rat leukocyte extracts. This line blended with that of the iron-binding protein precipitated by the unspecific goat antiserum. Further proof of the identity of this protein with lactoferrin was furnished by the finding that goat antiserum to human lactoferrin developed, in the leukocyte extract from rats, a precipitating line merging with the one formed by the monospecific rabbit antiserum (Fig. 7).

EXTRACTION OF LACTOFERRIN FROM THE LIVER, LUNG, KIDNEY, AND SPLEEN OF RATS INJECTED WITH ENDOTOXIN. Endotoxin (0.5 mg in 1-ml saline) was injected into the peritoneal cavity of six rats. After 6 h the animals were killed by bleeding. Their liver, spleen, lungs, and kidneys were removed, homogenized, frozen, and thawed six times, and extracted with 1 M saline. The insoluble fraction was discarded by centrifugation at 20,000 rpm for 50 min. The concentration of lactoferrin was determined by nephelometric immunoassay in the supernates after appropriate dilution.

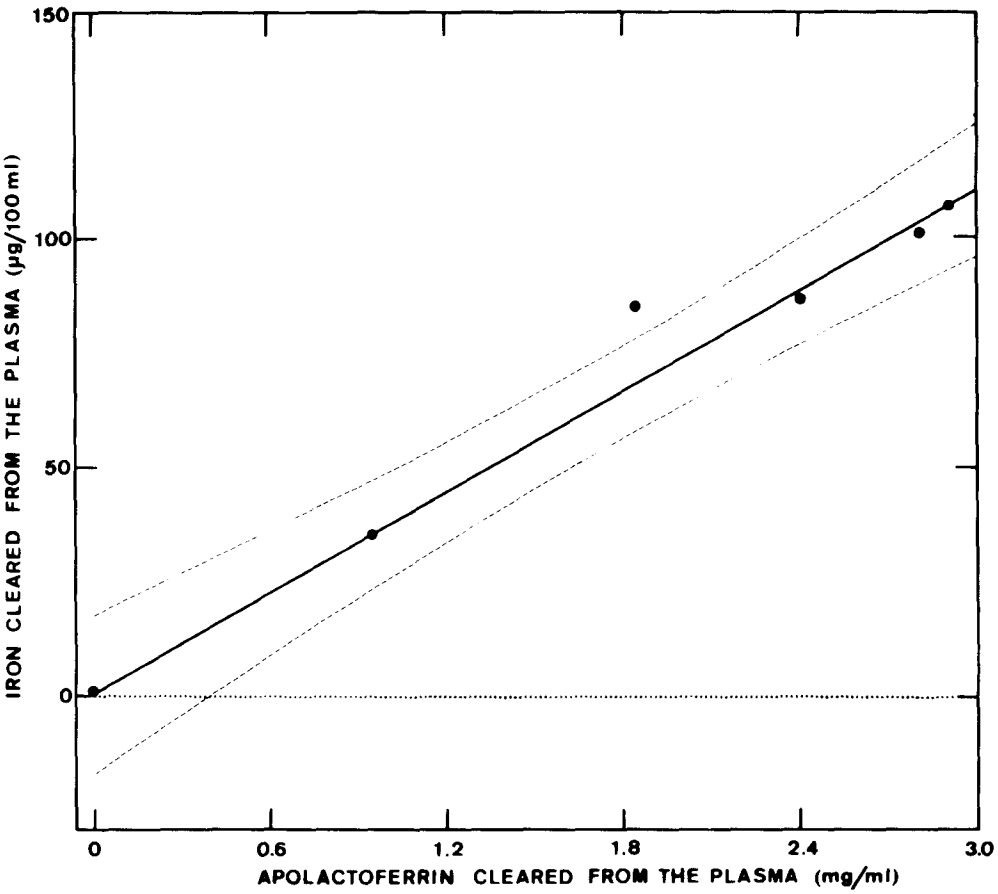

FIG. 3. Proportionality between the amounts of serum iron and apolactoferrin cleared from the plasma. The stippled lines delineate the ±2 SD range. The dots represent mean values from 3 to 10 individual animals.

The injection of endotoxin caused the plasma iron concentration to fall from 2.4 (SD, 0.2) to 1.2 (SD, 0.22) µg/ml. On the average, 2.6 mg of lactoferrin (SD, 0.34 mg) was recovered from the liver, 1.5 mg (SD, 0.29 mg) from the lungs, and 0.26 mg (SD, 0.04 mg) from the kidneys of the animals injected with endotoxin, whereas no lactoferrin was demonstrable by immunoelectrophoresis (Fig. 8) or immunonephelometry in the extracts of the liver or lungs of four normal rats. Some lactoferrin was detected in spleen extracts from normal rats (0.12 mg/spleen; SD, 0.03 mg) whereas twice that amount was present in the spleen of the animals injected with endotoxin. From these data it may be estimated that endotoxin is capable of causing the appearance of at least 4.3 mg of additional lactoferrin in the organs of the rat. When [^{59}Fe]citrate (5 µCi) was injected intravenously into rats 30 min before the administration of endotoxin, the isotope was found to be associated with the lactoferrin extracted from the lungs and liver, as shown by autoradiography of the immunoelectrophoreses, developed with rabbit antiserum against rat lactoferrin.

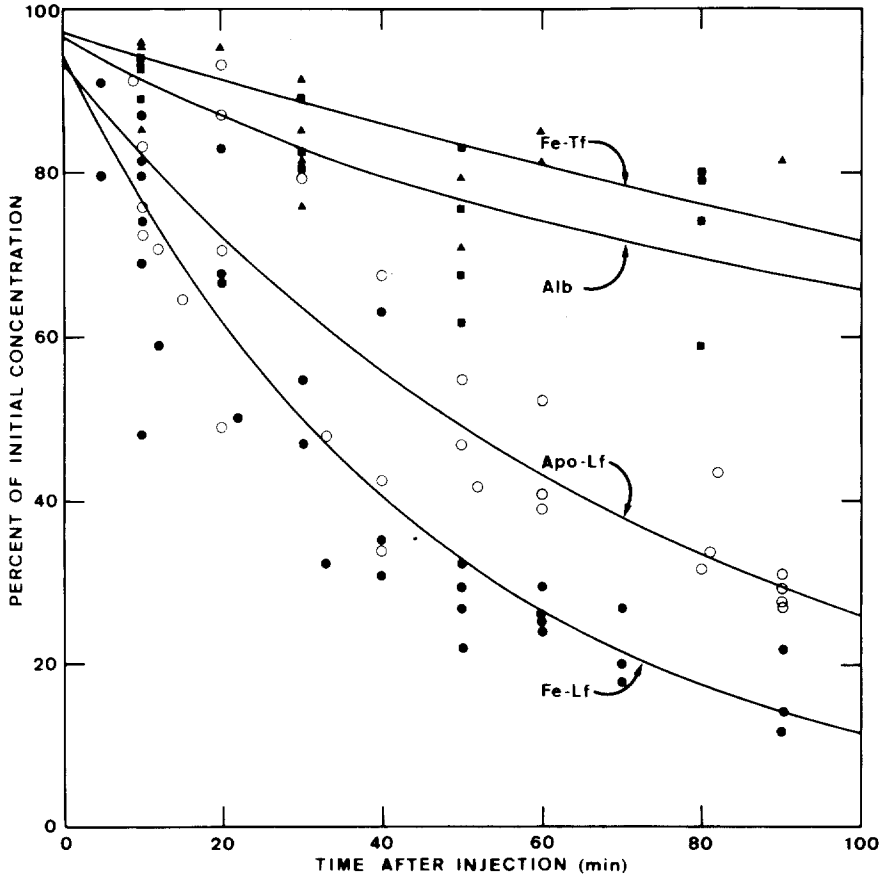

FIG. 4. Clearance of various proteins from the circulation in rats intravenously injected with 15 mg of, respectively: (▲), Fe-Tf, iron saturated human transferrin (3 rats); (○), Apo-Lf, human lactoferrin (87% iron-free—7 rats); (●), Fe-Lf, human lactoferrin saturated with iron (8 rats); and (■), Alb, human serum albumin (4 rats). Clearance curves (solid lines) were computed by least-square fitting to an exponential decay equation.

DETECTION OF LACTOFERRIN IN THE BLOOD OF RATS WITH A BLOCKED RES. Since lactoferrin was suspected to be eliminated by the RES, experiments were undertaken on seven rats whose RES had been blocked by three injections of India ink. Endotoxin (0.5 mg in 1 ml of saline) was injected intraperitoneally immediately after the last administration of colloid. The blood was collected 4 h later and the concentration of lactoferrin in the plasma was determined by nephelometric immunoassay. A mean concentration of 58 μg/ml (SD, 7) was found, whereas no lactoferrin (<10 μg/ml) was detectable in the plasma of three animals injected with endotoxin but with an intact RES, nor in that of normal, untreated rats. The lactoferrin revealed by immunoelectrophoresis of the plasma from animals with blocked RES showed an erratic mobility (Fig. 8) as it migrated in the α_2-region. This change in the electrostatic charge of lactoferrin was due to interaction with albumin, as shown by immunoelectrophoretic experiments on

TABLE IV

Intial Half-Lives of Various Human Proteins in Rats

Proteins	Initial half-life	SD	No. of rats
	min	*min*	
100% saturated lactoferrin	33	1.6	8
50% saturated lactoferrin	44	3	4
13% saturated lactoferrin	53	3.8	7
Succinylated Fe-lactoferrin	103	13.4	4
Fe-transferrin	243	73	3
Albumin	165	34	4
Fe-lactoferrin after blocking the RES with			
India ink	90	25	3
Aggregated albumin	110	26	3

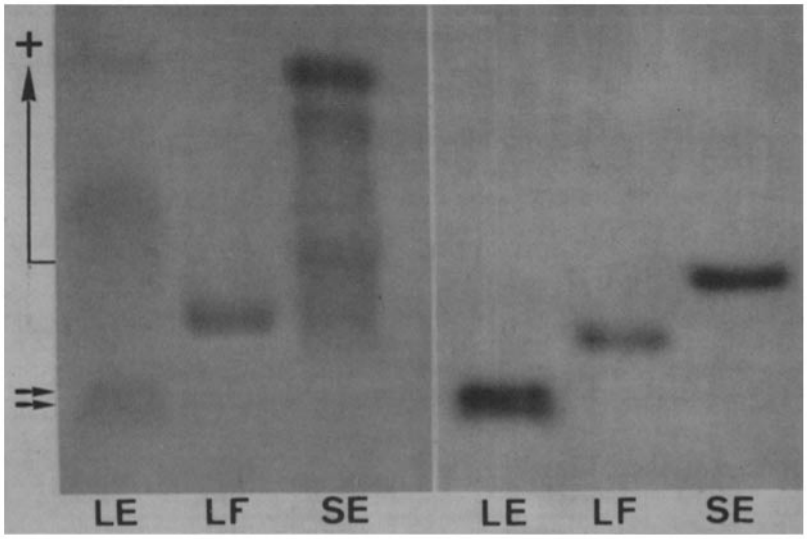

FIG. 5. Cellulose acetate electrophoresis (pH 8.6) of: LE, rat leukocyte extract; LF, human lactoferrin; and SE, rat serum. Left, stained for protein (Ponceau Red). Right, autoradiography. Arrows, the two lactoferrin components of rat leukocytes.

artificial mixtures containing different proportions of leukocyte extract and human serum albumin (see Fig. 8).

Discussion

From our in vitro experiments it appears that iron is effectively removed by apolactoferrin from transferrin only at pH values below 7, and that this exchange is greatly facilitated by the presence of chelators. It is generally admitted that

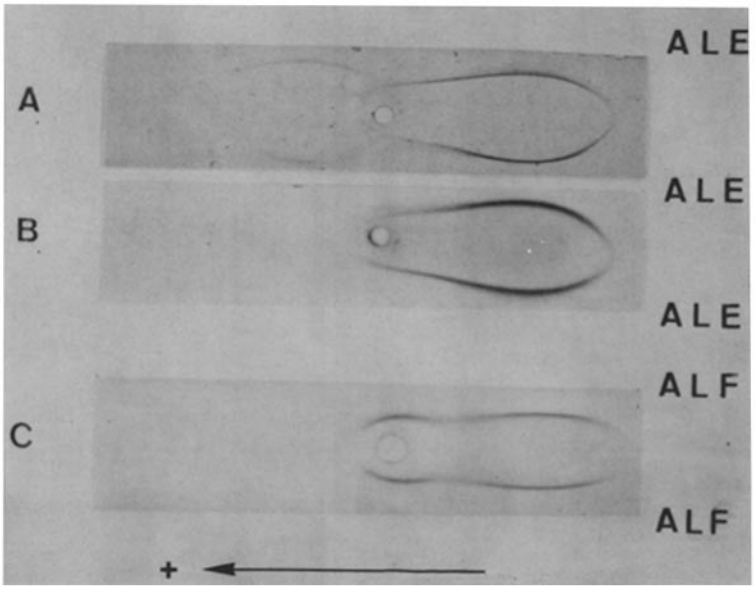

FIG. 6. Immunoelectrophoretic analyses of rat leukocytes extracts (A, B, C) developed with: ALE, goat antiserum to rat leucocyte extract; and ALF, rabbit antiserum to rat lactoferrin. A and C stained for protein (Amido Black) and B, autoradiography of A.

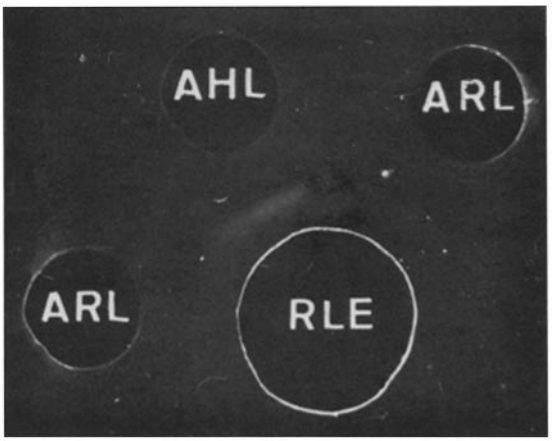

FIG. 7. Immunologic identification of rat leukocyte lactoferrin. RLE, rat leucocyte extract; ARL, rabbit antiserum to rat lactoferrin; and AHL, goat antiserum to human lactoferrin.

low pH levels prevail in inflamed tissues, due to the release of lactic acid by the accumulating stimulated polymorphonuclear leukocytes (28). Therefore, at such sites, e.g. in abscesses, one would expect an exchange of iron to occur between the Fe-transferrin adduced from the plasma and the apolactoferrin released by the leukocytes. The iron-chelating capacity of the lactic acid set free on such an occasion may conceivably assist in this process.

However, in systemic inflammatory reactions such as that caused by endotoxin, a lowering of the pH in any region of the organism has, to our knowledge,

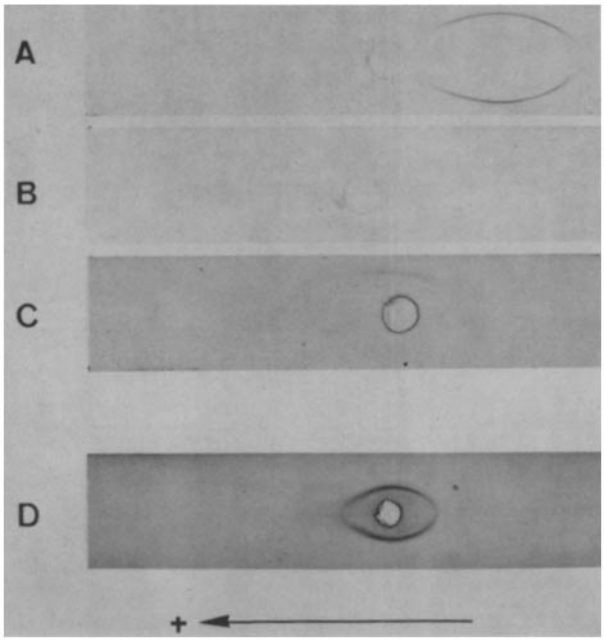

FIG. 8. Immunoelectrophoretic analyses, by antiserum to rat lactoferrin, of: A and B, liver extracts from endotoxin-treated (A) and untreated (B) rats; C, plasma from an endotoxin-treated rat with blocked RES; and D, rat leukocyte extract diluted in normal rat serum to reproduce the abnormally fast electrophoretic mobility of lactoferrin seen in C and due to interaction of lactoferrin with serum proteins.

never been reported. A change in pH seems even more unlikely in the hyposideremia caused by the injection of apolactoferrin. Nevertheless, as shown by our results and discussed hereafter, apolactoferrin originating from leukocytes is clearly the factor which removes the iron from the circulation. A plausible explanation may reside in the particularly high rate at which Fe-lactoferrin is cleared from the blood, together with the metal it has taken up. As a result, the equilibrium of the exchange reaction,

$$TfFe_2 + Lf \rightleftarrows Tf + LfFe_2,$$

is constantly shifted to the right, because, as soon as $LfFe_2$ is formed, this molecular species is preferentially removed. This is shown by a comparison of the clearance rates of Lf and $LfFe_2$ (Table IV). Equilibrium constants for the binding reactions of these two proteins at neutral pH are unfortunately not available, but it is known that in the pH 6.4–6.7 range, the equilibrium constant K_1 for Fe^{3+} is about 300 times larger for lactoferrin than for transferrin (29). Incidentally, the iron-chelating citrate molecule occurs in human plasma at the concentration of about 0.1 mmol/liter (30).

In regard to the fall in the iron content of the plasma that was induced by an injection of apolactoferrin, several convergent data indicate that this effect actually resulted from the direct action of exogenous apolactoferrin and not from an inflammatory reaction caused by the inadvertent introduction of some bacterial toxin contaminating the protein

solution: (*a*) A sample of lactoferrin from the same batch but saturated with iron did not change the plasma iron level. The iron present in the protein could not interfere with the determination, since, at the time of the assay most of the Fe-lactoferrin injected (97%) had already been eliminated. (*b*) Injections of proteins other than lactoferrin did not decrease the plasma iron level, which to the contrary was found to rise after the injection of apotransferrin. The latter finding could be explained by the capability of transferrin to mobilize storage iron (31). (*c*) It is known that the effect of an intraperitoneal dose of endotoxin on the serum iron level does not reach its maximum before 8–12 h (5). In contrast, the hyposideremia caused by exogenous apolactoferrin was already detectable 2 h after the injection. (*d*) Finally, the lowering of serum iron was linearly related to the amount of exogenous lactoferrin cleared from the blood (Fig. 3).

The specificity of the affinity of lactoferrin for macrophage membranes, shown by our data, must be scrutinized in the light of the knowledge that macromolecules with a high net-positive charge are preferentially bound and engulfed by these cells (32). However, the fact that lactoferrin was cleared faster in its iron-saturated form than when iron-free, seems to imply the existence of a specific receptor on the membrane of macrophages as well as a corresponding binding site on the molecule of lactoferrin. Succinylation, which was found to slow down the clearance of lactoferrin, presumably altered the regions involved in the binding reaction.

As mentioned in the introduction, the trapping of serum iron, under the form of ferritin, in inflammatory loci as well as in organs rich in reticuloendothelial cells, e.g. the liver and spleen, is a typical feature of inflammation (3, 4). In the light of our results, it seems probable that the lactoferrin-binding macrophage is the cell causally involved in this phenomenon. Whether Fe-lactoferrin constitutes a stimulus for the biosynthesis of apoferritin remains to be verified. What is clear from our data is that large amounts of Fe-lactoferrin appear in the liver, spleen, and lungs at a very early stage of the systemic inflammatory syndrome induced by the injection of endotoxin. Some of the lactoferrin extracted from these organs in our experiments doubtlessly originated from the disruption of the polymorphonuclear leukocytes that infiltrated the tissues due to the systemic inflammatory condition. However, this can only account for a mere fraction of the lactoferrin extractible from these tissues, since the average lactoferrin content of polymorphonuclear leukocytes, viz. 3 $\mu g/10^6$ cells (9), would require the presence of a far larger number of such cells in the organs involved than was actually seen in histological sections (unpublished observations). Moreover, this tissue lactoferrin was found to carry the radioactive iron previously present on serum transferrin, whereas leukocyte lactoferrin should have been essentially iron-free and should not have been able to take up the ^{59}Fe present in the preparation under the alkaline extraction conditions (pH 8.2) employed. All this leads to the conclusion that the major part of the tissue lactoferrin must have been present in or on macrophages. Immunohistological studies now in progress fully substantiate this view.

In conclusion, apolactoferrin released by neutrophilic leukocytes appears to be the agent responsible for the accumulation of iron in the RES during inflammation. Since the organism, for reasons still unknown, preferentially draws the iron needed for the biosynthesis of hemoglobin from destroyed erythrocytes rather than from the iron stores (33), the interference of lactoferrin with the metabolism of this metal might explain why inflammation causes hypochromic anemia.

Summary

The hyposideremia of inflammation was found to be based on a three-step mechanism involving lactoferrin, the iron-binding protein from the specific granules of neutrophilic leukocytes.

(*a*) *Lactoferrin is Released from Neutrophils in an Iron-Free Form.* When phagocytosis was induced in neutrophils by zymosan or bacteria, lactoferrin was recovered in the incubation medium together with other constituents of the specific granules, such as alkaline phosphatase and lysozyme. Lactoferrin extracted from leukocytes was able to bind the amount of iron corresponding to its theoretical iron-binding capacity. After injection of endotoxin into rats, lactoferrin was detected in various tissues where it was normally absent, or in the plasma when the reticuloendothelial system (RES) had previously been blocked by injections of India ink or aggregated albumin.

(*b*) *Lactoferrin is Able to Remove the Iron from Transferrin.* Significant exchange of iron from transferrin to lactoferrin was observed in vitro only at a pH below 7.0 or in the presence of a high concentration of citrate. However, the fast elimination of lactoferrin in vivo, when saturated with iron, might account for the observed transfer of iron to endogenous or administered apolactoferrin. Intravenous injection of human apolactoferrin into rats caused a marked decrease of the plasma iron level. The kinetics of this process, as well as controls with other proteins, ruled out the possibility of a secondary inflammatory effect due to phlogogenic contaminants.

(*c*) *Fe-Lactoferrin is Taken-up by the RES.* By immunofluorescence, lactoferrin was shown to be bound and ingested by monocytes. The rate of elimination of human Fe-lactoferrin injected into rats was particularly fast when compared to that of human apolactoferrin, succinylated Fe-lactoferrin, or other human proteins. Blockade of the RES slowed down the rate of clearance of Fe-lactoferrin and was also found to retard the elimination of endogenous rat lactoferrin released by endotoxin. These experiments suggest the existence of specific receptors for Fe-lactoferrin on the membrane of macrophages.

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Bibliography

1. Cartwright, G. E. 1966. The anemia of chronic disorders. *Semin. Hematol.* **3**:351.
2. Kampschmidt, R. F., and H. F. Upchurch. 1962. Effects of bacteria endotoxin on plasma iron. *Proc. Soc. Exp. Biol. Med.* **110**:191.
3. Cartwright, G. E., C. J. Gubler, and M. M. Wintrobe. 1950. The anemia of infection. XII The effect of turpentine and colloidal thorium dioxide on the plasma iron and plasma copper of dogs. *J. Biol. Chem.* **184**:579.
4. Wöhler, F., and W. Otte, 1961. Zur Pathologie des Speichereisens. II. Mitteilung: Ferritin- und Hämosiderin-gehalt menschlicher Organe bei entzündlichen Erkrankungen, Geschwülsten und Krankheiten des blutbildenden Systems. *Acta Haematol. (Basel).* **26**:81.

5. Kampschmidt, R. F., H. F. Upchurch, and H. L. Johnson. 1965. Iron transport after injection of endotoxin in rats. *Am. J. Physiol.* **208**:68.
6. Kampschmidt, R. F., and H. F. Upchurch. 1969. Lowering of plasma iron concentration in the rat with leukocytic extracts. *Am. J. Physiol.* **216**:1287.
7. Pekarek, R. S., and W. R. Beisel. 1971. Characterization of the endogenous mediator(s) of serum zinc and iron depression during infection and other stresses. *Proc. Soc. Exp. Biol. Med.* **138**:728.
8. Masson, P. L. 1970. La Lactoferrine. Maloine (Librairie), S. A., Paris, France.
9. Masson, P. L., J. F. Heremans, and E. Schonne. 1969. Lactoferrin, an iron-binding protein in neutrophilic leukocytes. *J. Exp. Med.* **130**:643.
10. Baggiolini, M., C. de Duve, P. L. Masson, and J. F. Heremans. 1970. Association of lactoferrin with specific granules in rabbit heterophil leukocytes. *J. Exp. Med.* **131**:559.
11. Johansson, B. 1960. Isolation of an iron-containing red protein from human milk. *Acta Chem. Scand.* **14**:510.
12. Montreuil, J., J. Tonnelat, and S. Mullet. 1960. Préparation et propriétés de la lactosidérophiline (lactotransferrine) du lait de femme. *Biochim. Biophys. Acta.* **45**:413.
13. Blanc, B., and H. Isliker. 1961. Isolement et caractérisation de la protéine rouge sidérophile du lait maternel: la lactoferrine. *Helv. Physiol. Pharmacol. Acta.* **19**:C13.
14. Querinjean, P., P. L. Masson, and J. F. Heremans. 1971. Molecular weight, single-chain structure and amino-acid composition of human lactoferrin. *Eur. J. Biochem.* **20**:420.
15. Masson, P. L., and J. F. Heremans. 1968. Metal-combining properties of human lactoferrin (red milk protein). I. The involvement of bicarbonate in the reaction. *Eur. J. Biochem.* **6**:579.
16. Masson, P. L., B. Teuwissen, J. Van Snick, T. K. Roberts, J. F. Heremans, and R. C. Topp. 1973. New data on lactoferrin, the iron-binding protein from external secretions and neutrophilic leucocytes. International Conference on Iron Storage and Transport Proteins. University College Hospital, Medical School, London, England. Abstr. 15.
17. Blanc, B., and H. Isliker. 1963. Transfert et équilibre de dialyse du ^{59}Fe entre les principales ferriprotéines. *Helv. Chim. Acta.* **322**:2905.
18. Lane, R. S. 1967. Localization of transferrin in human and rat liver by fluorescent antibody technique. *Nature (Lond.).* **215**:161.
19. Masson, P. L., J. F. Heremans, and C. Dive. 1966. An iron-binding protein common to many external secretions. *Clin. Chim. Acta.* **14**:735.
20. Hirsch, J. G. 1956. Phagocytin: a bactericidal substance from polymorphonuclear leukocytes. *J. Exp. Med.* **103**:589.
21. Mancini, G., A. O. Carbonara, and J. F. Heremans. 1965. Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochemistry.* **2**:235.
22. Ritchie, R. F., C. A. Alper, M. A. Graves., N. Pearson, and C. Larson. 1973. Automated quantitation of proteins in serum and other biologic fluids. *Am. J. Clin. Pathol.* **59**:151.
23. Cambiaso, C. L., J.-P. Vaerman, P. L. Masson, and J. F. Heremans. 1974. Automated nephelometric immunoassay (ANIA). I. Importance of antibody affinity. *J. Immunol. Methods.* **5**:153.
24. Schumacher, G. F. B., and M. J. Pearl. 1969. Cyclic change of muramidase (lysozyme) in cervical mucus. *J. Reprod. Med.* **3**:171.
25. Biozzi, G., B. Benacerraf, and B. N. Halpern. 1953. Quantitative study of the granulopectic activity of the reticuloendothelial system. II. A study of the kinetics of the granulopectic activity of the RES in relation to the dose of carbon injected.

Relationship between the weight of the organs and their activity. *Br. J. Exp. Pathol.* **34**:441.

26. Masahiro, H. O., and H. N. Wagner. 1962. Studies of the reticuloendothelial system (RES). I. Measurement of the phagocytic capacity of the RES in man and dog. *J. Clin. Invest.* **42**:417.
27. Bates, G. W., C. Billups, and P. Saltman. 1967. The kinetics and mechanism of iron (III) exchange between chelates and transferrin. I. The complexes of citrate and nitrilotriacetic acid. *J. Biol. Chem.* **242**:2810.
28. Policard, A. 1965. Les réactions inflammatoires et leur dynamique. Masson et Cie, Paris, France.
29. Aisen, P., and A. Leibman. 1972. Lactoferrin and transferrin: a comparative study. *Biochim. Biophys. Acta.* **257**:314.
30. Natelson, S., J. B. Pincus, and J. K. Lugovoy. 1948. Response of citric acid levels to oral administration of glucose; normal adults and children. *J. Clin. Invest.* **27**:446.
31. Finch, C. A., K. Deubelbeiss, J. D. Cook, J. W. Eschbach, L. A. Harker, D. D. Funk, G. Marsaglia, R. S. Hillman, S. Slichter, G. W. Adamson, A. Ganzoni, and E. R. Giblett. 1970. Ferrokinetics in man. *Medicine (Baltimore).* **49**:17.
32. Yokomura, E. 1969. Induction of phagocytosis of iron colloid by Ehrlich ascites tumor cells with polycationic substances. *Gann.* **60**:439.
33. Noyes, W. D., T. H. Bothwell, and C. A. Finch. 1960. The role of the reticuloendothelial system in iron metabolism *Br. J. Haematol.* **6**:43.