THE BINDING OF HUMAN LACTOFERRIN TO MOUSE PERITONEAL CELLS*

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Lactoferrin (Lf)[1] is a glycoprotein which, like transferrin (Tf), reversibly binds two ferric ions with the incorporation of two molecules of bicarbonate. These two proteins have a mol wt of 76,000 and are clearly related by their amino acid sequence (1-6). Also both proteins display a clear bacteriostatic activity by depriving the medium of the iron necessary for the growth of bacteria (7-9).

However, Lf and Tf differ on several points. They fail to show any immunological cross-reaction unless denatured (10). Further, the affinity of Lf for iron is largely retained at pH values below 4.0, whereas under such conditions Tf completely releases its iron (1-3).

Tf is a plasma protein which is synthesized mainly in the liver (11). Lf occurs in the plasma only in trace amounts (12, 13) but abounds in external secretions (14) and specific granules of neutrophils (15, 16). Yet another difference resides in the ability of Tf to combine with the membrane of reticulocytes which possess a receptor specific for Tf (17), which is inaccessible to Lf (18). In a previous paper (19), we have shown that Lf has a particular affinity for the reticuloendothelial system (RES), and that this property could explain the involvement of this protein in the hyposideremia of inflammation.

The present work provides experimental data suggesting the existence of a specific receptor for Lf on mouse peritoneal cells (MPC) and, more particularly, on macrophages. Ideally a study of interaction of protein and cells from one species would provide the best experimental model. However, the difficulties of obtaining sufficient amounts of both protein and cells from the same species imposed on us the heterologous system of human Lf and mouse cells.

Materials and Methods

Reagents. Human Lf was isolated from milk by chromatography on carboxymethyl Sephadex (5). Somes batches were prepared after saturation of the protein (FeLf) by adding ferric citrate in the presence of bicarbonate (4). One sample was isolated in a form poor in iron (18% saturation calculated from the absorbance at 460 nm). The latter sample will be referred to as apoLf. A sample of FeLf was deprived of its iron by dialysis against 0.1 M citric acid (5).

Human Tf devoid of iron was obtained from Behring-Institut, Marburg/Lahn, W. Germany. Human IgG was prepared from a pool of normal sera by precipitation with Rivanol (6,9-diamino-

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[1] *Abbreviations used in this paper:* BME, basal medium of Eagle; BSA, bovine serum albumin; Lf, lactoferrin; MPC, mouse peritoneal cells; RES, reticuloendothelial system; Tf, transferrin.

acridine lactate provided by Federa, Brussels). A sample of IgG was aggregated by heating a 1% solution at 63°C for 30 min. Beef heart cytochrome *c* was purchased from Sigma Chemical Co., St. Louis, Mo. Ficoll was provided by Pharmacia Fine Chemicals, Uppsala, Sweden, and Isopaque by Nyegaard & Co., Oslo, Norway.

Cells. Peritoneal cells were collected according to the procedure of Cohn and Benson (20). A vol of 4 ml basal medium of Eagle (BME) containing 2 mg/ml heparin was injected into the peritoneal cavity of NMRI female mice weighing about 30 g, and reaspirated after abdominal kneading. On the average, 5×10^6 MPC were harvested from one animal. They consisted of about 70% macrophages and 30% lymphocytes.

Suspensions of mouse spleen cells in BME were centrifuged on a Ficoll Isopaque mixture (ρ = 1,077) for 40 min at 400 *g*. After centrifugation, the cells present at the interface between the Ficoll Isopaque and the medium were washed twice in BME and finally resuspended in BME containing 1% bovine serum albumin (BSA, provided by Calbiochem, San Diego, Calif.) before incubation with FeLf. These cells consisted of more than 90% lymphocytes. L1210 mouse lymphoma cells are maintained in culture in our laboratory.

Methods. Succinylation of FeLf was carried out by treating 50 mg protein in 3 ml of 0.2 M NaCl and 0.1 M $NaHCO_3$ with 25 mg of succinic anhydride at room temperature for 1 h. The pH was maintained at 8.5 by addition of 0.1 M NaOH.

For carbamylation, 10 mg FeLf was incubated in phosphate-buffered saline, pH 7.2, at 45°C with 1 M potassium cyanate. Various samples of FeLf differing by their degree of chemical modification were prepared by changing the time of incubation. The reaction was stopped by passing the reagents through a column of Sephadex G-25.

To remove neuraminic acid from FeLf, 50 mg protein dissolved in 5 ml of sodium acetate buffer was incubated at 37°C for 48 h with 500 U of neuraminidase from *Vibrio cholerae* (Behring-Institut). The solution was then dialyzed for 24 h against 0.05 M ammonium bicarbonate, and lyophilized. Previous experiments (21) had shown that after such a treatment no sialic acid remained on FeLf. This solution will be referred to as NANA-free FeLf. ^{59}FeLf and ^{59}FeTf were prepared by adding ^{59}Fe-citrate to the apoproteins, in the presence of bicarbonate, up to about 80% of their binding capacities. Tf and Lf were iodinated with carrier-free ^{125}I using the chloramine T technique (22). Free iodine was separated from the protein by gel filtration on Sephadex G-25. Iodine-labeled proteins were stored at −20°C.

In the first experiments, the amount of protein bound by the cells was calculated according to the method of Arend and Mannik (23), where cells were repeatedly washed and corrections made for nonspecific adsorption. However, in our experiments with Lf, after having applied the Arend and Mannik method, we realized that only a single washing of the pellet and the tube without cells followed by substraction of the counts detected in the tubes without MPC were required. Therefore, in further experiments, the amount of Lf specifically bound to MPC was determined by this simplified method.

Results

Kinetics of Lf Binding to MPC. Peritoneal cells were incubated at 37°C with ^{125}I- or ^{59}Fe-labeled FeLf in BME containing 1% BSA. At various time intervals, the reaction was stopped by diluting the reagents 10 times with BME containing BSA. The cells were immediately centrifuged at 100 *g* for 5 min at 4°C, and the radioactivity of the pellet counted (Fig. 1). After 30 min, the binding curve tended to flatten. No significant difference in the binding was observed between the ^{59}Fe- or ^{125}I-labeled preparations.

To see whether FeLf was adsorbed onto the membranes or ingested, the cells were incubated for 30 min at either 37°C or 4°C with ^{59}FeLf (Fig. 2). No significant difference was observed, suggesting that the protein was only bound to the cell membrane.

Binding of Lf to MPC as a Function of Lf Concentration. In a series of seven experiments, ^{59}FeLf was added in increasing amounts to MPC and incubated at 37°C for 30 min. After centrifugation and washing, the radioactivity was mea-

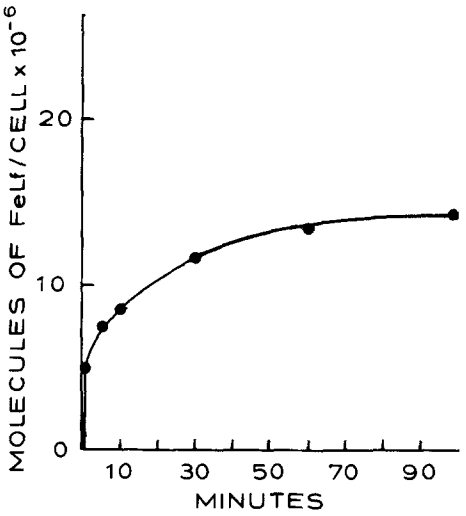

FIG. 1. Binding of ^{59}FeLf to MPC as a function of time. Each point represents the mean of three experiments.

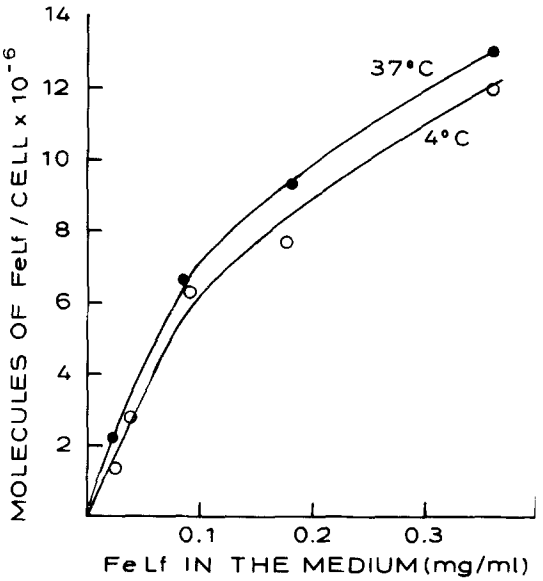

FIG. 2. Binding of FeLf to MPC at 37°C or 4°C. Each point represents the mean of three experiments.

sured in the pellet and the results corrected for the residual amount of free FeLf contaminating the cells. MPC were saturated with FeLf when its concentration in the medium reached, on the average, 300 μg/ml (Fig. 3).

A similar experiment was performed with ^{125}I-labeled human Tf, which was half-saturated with iron. No binding to MPC was observed.

The Inhibition of Binding of FeLf to MPC. To study the specificity of the binding of FeLf to MPC, we have compared the amount of ^{59}Fe- or ^{125}I-labeled FeLf bound to the cells after their incubation with various proteins at different

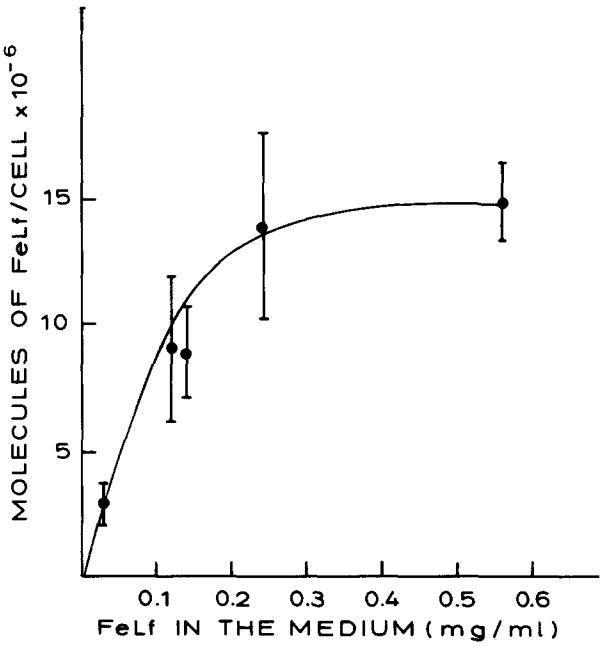

FIG. 3. Saturation curve of MPC with increasing amounts of FeLf. Each point represents the mean of seven experiments and the vertical bar, one standard deviation.

concentrations. MPC were preincubated with the potential inhibitor at 4°C for 15 min in BME containing 1% BSA. Then, 500 μg of radioactive FeLf was added, and the incubation prolonged for another 30 min. The percent of inhibition was plotted vs. the concentration of the competitive protein (Fig. 4). At the same concentration of both reagents, unlabeled FeLf inhibited by 50% the binding of radioactive FeLf. Human IgG did not interfere significantly. NANA-free FeLf displayed an inhibitory activity clearly higher than native FeLf.

A second series of inhibition experiments was carried out using only one concentration of the competitive protein, i.e., 500 μg/ml (Table I). ApoLf inhibited the binding of radioactive FeLf to a lower extent than did cold FeLf. The difference was at the limit of statistical significance ($0.05 < P < 0.10$). Surprisingly, when the sample of Lf which was deprived of its iron by dialysis against citric acid was used, no inhibition was observed. Furthermore, no inhibitory activity was recovered even when iron was added to this Lf sample, of which the iron saturation was shown by the reappearance of the characteristic pink hue.

After succinylation or carbamylation, Lf retained its iron as shown by its absorbance spectrum. However, the protein lost its inhibitory activity as a reciprocal function of its chemical modification. Various samples of carbamylated FeLf were analyzed in electrophoresis in agarose gel at pH 8.6 together with native FeLf and sialic acid-free FeLf. When the inhibitory activity of each preparation was plotted vs. their electrophoretic mobility, a linear relationship was observed (Fig. 5), indicating that the affinity of FeLf for mouse peritoneal cells decreased with the suppression of positive electric charge of the protein. However, a protein with a high isoelectric point such as beef heart cytochrome *c* (pI = 10.6, reference 24) failed to inhibit the binding of FeLf.

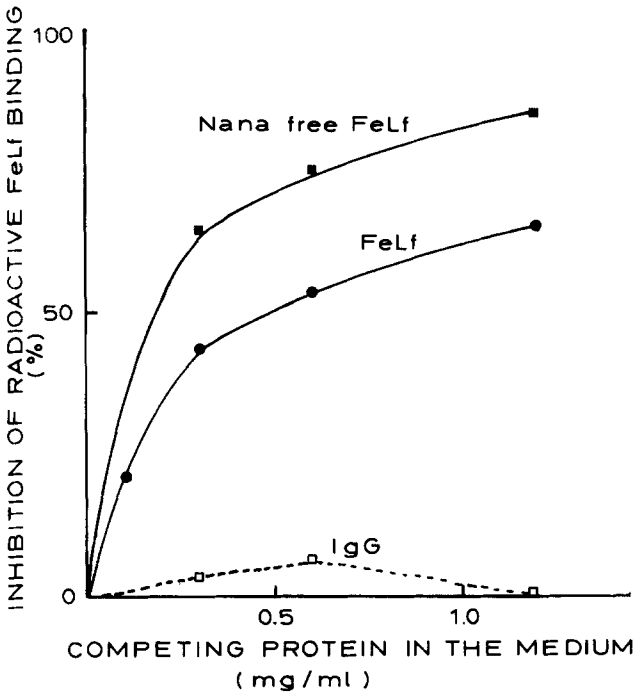

FIG. 4. Inhibition of the binding of ^{125}I-labeled FeLf to MPC by various human proteins. Each point represents the mean of three experiments.

TABLE I

Inhibition of the Binding of Radioactive FeLf to MPC by Various Proteins

Competitive protein	Percent of inhibition (Mean ± SD)	
	^{59}Fe-labeled FeLf	^{125}I-labeled FeLf
FeLf	46.4 ± 7.4 (13)	46.0 ± 8.1 (6)
ApoLf	28.2 ± 4.9 (3)	
Fe-deprived Lf	No inhibition (3)	
Fe-resaturated Lf	No inhibition (3)	
NANA-free FeLf	53.0 ± 8.3 (4)	66.6-62.8
Succinylated FeLf	No inhibition (4)	
Carbamylated FeLf*		
1 min	40.8 ± 2.4 (4)	
5 min	31.7 ± 2.0 (4)	
10 min	20.0 ± 5.2 (4)	
30 min	No inhibition (4)	
FeTf	9.1 ± 5.8 (5)	14.7-14.9
Human IgG	No inhibition (4)	6.6 ± 2.4 (3)
Human aggregated IgG	No inhibition (5)	
Beef heart cytochrome *c*	No inhibition (4)	

The figures in parenthesis correspond to the number of experiments.

* The time mentioned refers to the incubation of FeLf with KCNO (see Materials and Methods).

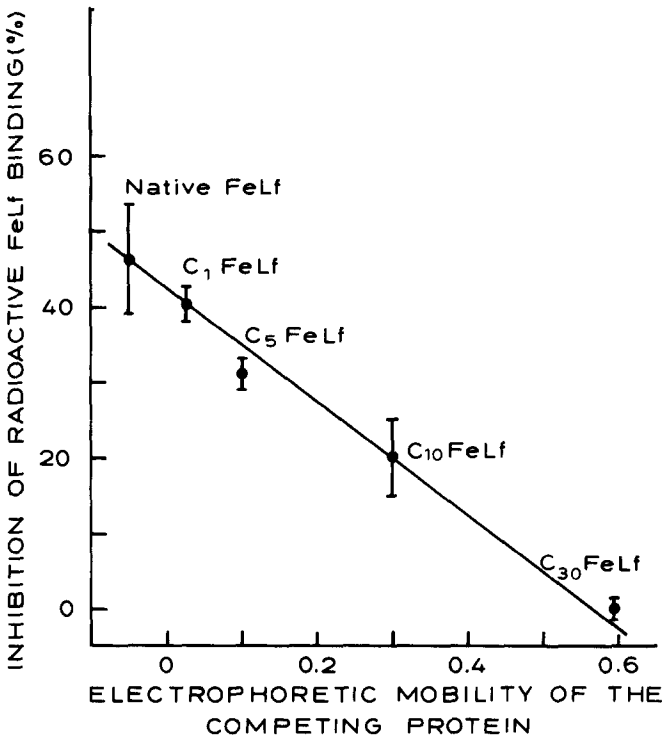

FIG. 5. Relationship between the inhibition of the binding of ^{125}I-labeled FeLf or ^{59}FeLf to MPC by cold FeLf or carbamylated FeLf, and the relative electrophoretic mobilities of these samples in agarose gel at pH 8.6. These relative mobilities were calculated by dividing the migration distance of the competing protein by that of human serum albumin. For carbamylation, FeLf was incubated with KCNO for increasing times. C_1 FeLf corresponds to 1-min incubation, C_5 FeLf to 5 min, C_{10} FeLf to 10 min, and C_{30} FeLf to 30 min. Each point represents the mean of four experiments and the vertical bars, one standard deviation.

To confirm that apoLf had a lower affinity towards the peritoneal cells than FeLf, the experiments of competition between FeLf and apoLf were resumed using two kinds of labeled reagents, i.e., ^{59}FeLf- and ^{125}I-labeled apoLf (Table II). 75% inhibition of ^{125}I-apoLf binding was obtained with FeLf. This result was significantly higher than the 51.6% inhibition resulting from the preincubation with apoLf ($0.001 < P < 0.01$). ApoLf inhibited the binding of ^{59}FeLf at a rate of 32.2%, whereas cold FeLf reached 56.3%. This difference was also significant ($0.02 < P < 0.05$).

Comparison of the Binding of FeLf to Various Mouse Cells. ^{125}I-labeled FeLf was added in constant concentration (100 μg per ml) to an increasing number of mouse cells from different origins, i.e. peritoneal cells, isolated spleen lymphocytes, and L1210 lymphoma cells. The radioactivity associated with the pellet was calculated after washing and appropriate corrections (see Materials and Methods). A linear relationship was observed between the amount of cell-associated Lf and the number of cells (Fig. 6). The slopes that indicated the number of FeLf molecules bound per cell differed widely depending on the type

TABLE II
Inhibition of the Binding of ^{125}I-apoLf or ^{59}FeLf to MPC by apoLf and FeLf

Competitive protein	Percent of inhibition	
	^{125}I-apoLf	^{59}Fe-Lf
	%	
ApoLf	51.6 ± 4.9 (5)	32.2 ± 4.3 (5)
FeLf	75 ± 2.3 (5)	56.3 ± 7.3 (4)

The figures in parentheses correspond to the number of experiments.

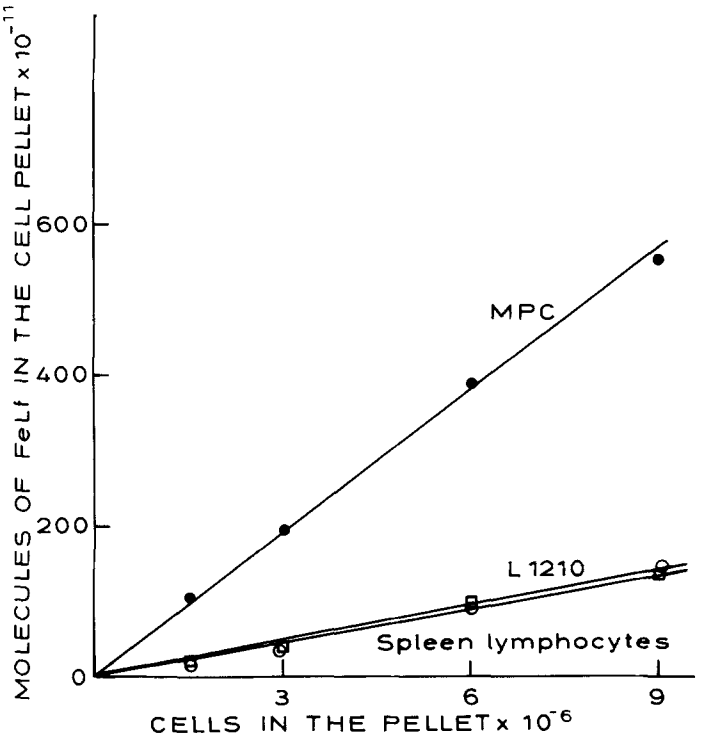

FIG. 6. Relationship between the amount of Lf associated with MPC, spleen lymphocytes, and L1210 cells, and the number of cells exposed to the protein.

of cells studied. The peritoneal cells bound much more FeLf than did spleen lymphocytes or L1210 lymphoma cells.

As a control, sheep and human red blood cells were incubated with the same preparation of ^{125}I-labeled FeLf under identical conditions. No binding at all was detected with these cells.

The Parameters of the Binding of Lf to Peritoneal Macrophages and Lymphocytes. The MPC were fractionated on a column of glass beads in order to separate the macrophages from lymphocytes. The macrophages, which adhered to the glass, were recovered by elution with 10 mM Na-EDTA in balanced salt solution without Ca^{++} and Mg^{++}. The viability of these macrophages was

checked by trypan blue exclusion. On the average, 20% of these cells were dead, whereas less than 4% of the lymphocytes that freely passed through the column failed to exclude the dye. Owing to the poor yield of viable macrophages, the study of the parameters of Lf binding was restricted to unfractionated MPC and to purified peritoneal lymphocytes. Knowing the quantity of Lf that these lymphocytes could retain, it was possible to calculate the quantity of Lf bound to the other cells constituting the MPC, i.e., the macrophages (Fig. 7). About 10% of FeLf was bound to lymphocytes.

A Schatchard plot was constructed for the binding of ^{125}I- of ^{59}Fe-labeled FeLf to lymphocytes and macrophages (Table III and Fig. 8). The experiments were carried out on the same cells, with a mixture of ^{125}I- and ^{59}Fe-labeled FeLf. No significant difference was observed between the affinity constant of FeLf toward macrophages or lymphocytes whatever the mode of labeling the proteins. However, the macrophages bound approximately three times more Lf than did the lymphocytes.

Discussion

The present work has shown that human Lf combines with the membranes of various mouse cells: macrophages, lymphocytes, and L1210 lymphoma cells. Because of the important role of macrophages in iron metabolism, we have studied mainly the interaction of Lf with these cells.

The existence of a specific receptor for Lf on the membrane of MPC is supported by the following data.

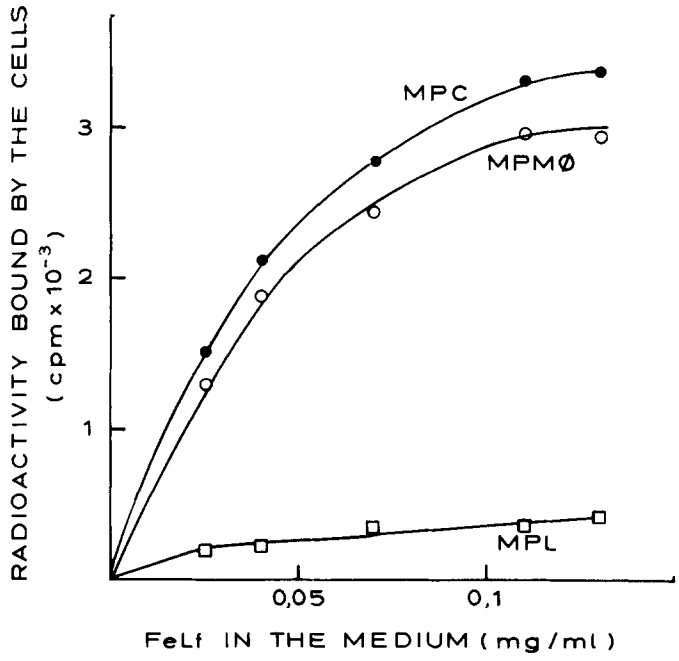

FIG. 7. Saturation curves of MPC and isolated mouse peritoneal lymphocytes (MPL) with increasing amounts of FeLf. The saturation curve of mouse peritoneal macrophages (MPMΦ) was obtained by subtracting the curve of MPL from that of MPC.

TABLE III

Number of Molecules (N) of ^{125}I- or ^{59}Fe-Labeled FeLf Bound to Mouse Peritoneal Lymphocytes or Macrophages, and the Affinity Constant (K) of This Association

Cells	^{125}I-FeLf			^{59}FeLf		
	Number of experiments*	K 10^6 liters/mol	N 10^6 per cell	Number of experiments*	K 10^6 liters/mol	N 10^6 per cell
Macrophages	7	0.67 ± 0.14	22.1 ± 6.2	5	0.93 ± 0.15	17.5 ± 2.1
Lymphocytes	3	0.95 ± 0.51	6.94 ± 2.49	5	1.06 ± 0.42	5.65 ± 2.55

* Each experiment consisted of 15 different determinations performed on a pool of MPC.

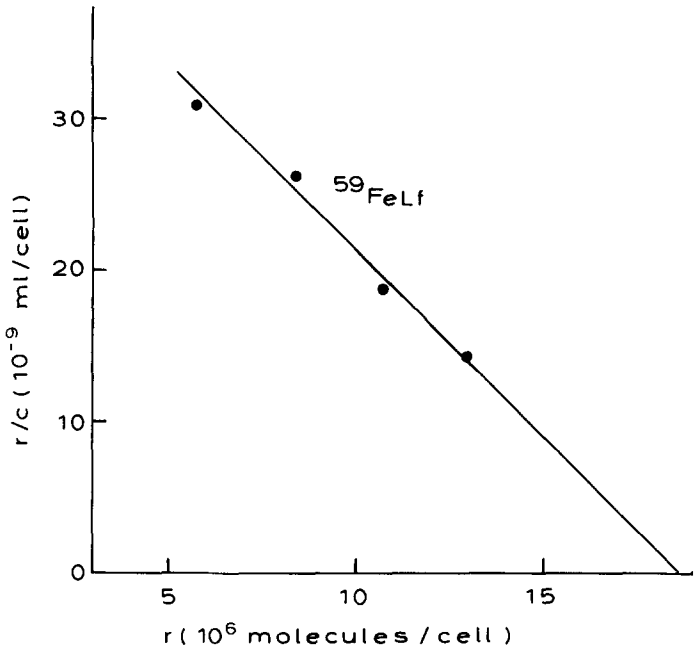

FIG. 8. Schatchard plot (r/c = nK − rK) of the binding of FeLf to mouse peritoneal macrophages. The molecules of Lf adherent to one cell (r) and the molecules of Lf free in the medium (c) were calculated using a mol wt of 76,000 for Lf. The value n, or the X-intercept, represents the number of receptors per cell, assuming one Lf molecule occupies one receptor site. The slope of the line, K, represents the effective association constant in liters/mol.

(*a*) The binding of FeLf to MPC reached a saturation point, which suggests that these cells possess a restricted number of binding sites.

(*b*) Other proteins such as human Tf, human aggregated as well as nonaggregated IgG, and beef heart cytochrome *c* failed to inhibit the binding of radioactive FeLf by MPC, whereas the reaction was inhibited by cold FeLf.

(*c*) The integrity of the structure of Lf was critical for its reaction with MPC. Succinylation, carbamylation, and even dialysis against 0.1 M citric acid resulted in a marked decrease in the affinity of the protein for the cells. This property of FeLf to combine with the cell membrane is much more sensitive to structural changes than the ability of the protein to bind iron since, after dialysis against citric acid, the protein was still able to bind iron, but the

complex failed to react with MPC. Similar observations were reported by Schade (25) when he studied the combination of Tf with the membrane of reticulocytes. This author noticed that certain samples of Tf easily lost their ability to react with reticulocytes while keeping their iron-binding capacity. Further experiments with carbamylated FeLf and NANA-free FeLf revealed the importance of the electric charge of the protein for its binding to MPC. It is known that Lf tends to form complexes with many acidic molecules such as agaropectin, trypan blue (26), and albumin (27). Hence, the high isoelectric point (pI = 9.0) of Lf (21) could in part account for the reaction of FeLf with MPC. However, basic nature of the protein is not the only requirement for binding, since beef heart cytochrome *c*, which has a pI of 10.6 (24), did not inhibit the binding of FeLf.

(*d*) The ability of apoLf to inhibit the binding of FeLf to MPC was significantly lower than that of FeLf. This could be explained by the steric changes that Lf undergoes when it combines with iron. The molecule of FeLf is more compact and more asymetric than that of ApoLf (5).

(*e*) Both sheep and human red blood cells did not bind Lf, whereas lymphocytes and leukemic cells were capable of binding Lf, albeit to a lower extent than do macrophages (Fig. 6 and 7). Peritoneal macrophages were found to bind on the average 20×10^6 molecules of FeLf per cell, whereas peritoneal lymphocytes bound only 6.7×10^6 molecules per cell. The calculated values for the affinity constants of Lf for macrophages and lymphocytes (0.9×10^6 liters/mol is of the same order of magnitude as the affinity constant of monomeric IgG for macrophages (23). However, the numbers of bound IgG molecules is 10 times lower than that of Lf molecules. Apparently, there is no relationship between the Fc and Lf receptors of macrophages, since monomeric as well as aggregated IgG failed to inhibit the binding of Lf.

The present work confirms and extends our previous observations that FeLf has a high affinity for the membrane of macrophages (18, 19). Previous experiments had shown that human Lf which was injected into rats was quickly removed by the RES with a clearance rate dependent upon the iron content of the protein and its structural integrity. The plasma half-life of apoLf was significantly prolonged, as well as that of succinylated FeLf, when compared to that of intact FeLf. Furthermore, after blockade of the RES, the half-life of FeLf was markedly increased.

The presence of a Lf receptor on macrophages is of interest as it could explain certain features of iron metabolism, particularly the hyposideremia of inflammation. This phenomenon is classically explained by the impairment of the reutilization of iron from hemoglobin catabolized in the RES. There is apparently a blockade in the release of iron from the RES. Fillet et al. (28) have shown that, in dogs, after the catabolism of hemoglobin in the RES, the iron returns to the plasma via two pathways, one very fast ($t^1/_2$, 24 min) and the other very slow ($t^1/_2$, 7 days). These pathways are of equal magnitude in normal animals, while in an acute inflammatory reaction there is a marked increase in the proportion of iron processed through the slow pathway. We propose that Lf, which is released from neutrophils, is adsorbed onto the membrane of macrophages. There it takes the iron released from these cells and immediately the FeLf complex is reintroduced into the RES where it accumulates in the form of

ferritin, as will be described elsewhere. This storage could correspond to the slow pathway described by Fillet et al. (28).

The presence of Lf on the membrane of macrophages must also be considered in the light of the ability of this protein to inhibit the growth of bacteria by competing for the iron necessary for bacterial development (7, 8). This activity, which is common to Lf and Tf, would be reinforced in the presence of antibodies (9). At present no role can be attributed to the binding of Lf to lymphocytes.

Summary

Human iron-saturated Lf (FeLf), which was labeled with ^{125}I or ^{59}Fe, was found to combine with the membrane of mouse peritoneal cells (MPC) which consisted of 70% macrophages. The following experimental data suggested the involvement of a specific receptor. (*a*) The binding of FeLf to MPC reached a saturation point. (*b*) The binding of radioactive FeLf was inhibited by preincubating the cells with cold FeLf but not with human Tf, human aggregated and nonaggregated IgG, or beef heart cytochrome *c*. (*c*) Succinylation and carbamylation of FeLf resulted in a loss of its inhibiting activity on the binding of radioactive FeLf. Removal of neuraminic acid from FeLf increased its inhibitory activity. (*d*) The ability of apoLf to inhibit the binding of FeLf to MPC was significantly lower than that of FeLf.

The existence of a Lf receptor capable of concentrating Lf released from neutrophils on the membrane of macrophages could explain the apparent blockade of the release of iron from the reticuloendothelial system, which accounts for the hyposideremia of inflammation. A receptor for FeLf was also found on mouse peritoneal lymphocytes. The affinity constant of FeLf for both lymphocytes and macrophages was 0.9×10^6 liters/mol. However, macrophages bound three times more FeLf molecules (20×10^6) per cell than did lymphocytes (7×10^6).

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