

# THE INGESTION AND DIGESTION OF HUMAN LACTOFERRIN BY MOUSE PERITONEAL MACROPHAGES AND THE TRANSFER OF ITS IRON INTO FERRITIN\*

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In a previous paper (1) we have reported results which indicated that lactoferrin (Lf),<sup>1</sup> the iron-binding protein from the specific granules of neutrophilic leukocytes might be involved in the hyposideremia occurring during inflammation. The role of Lf in this phenomenon is related to the high affinity of Lf for iron (2) and the existence of a receptor for this protein on the membrane of macrophages (3).

In the present work, we have further investigated the Lf-macrophage interaction in relation to the ingestion of the protein, its intracellular fate, and finally, the transfer of iron from Lf into ferritin.

## Materials and Methods

*Cells.* Mouse peritoneal macrophages (MPM) were harvested from NMRI female mice by rinsing the peritoneal cavity with 5 ml of basal medium of Eagle (BME), containing 10 U/ml of heparin and 50  $\mu$ g/ml of streptomycin. After centrifugation at 100 *g* for 10 min, the cells were resuspended in BME supplemented with 10% heat inactivated fetal calf serum (FCS) and with penicillin 50 U/ml and streptomycin (50  $\mu$ g/ml), and distributed in Linbro tissue culture plates (Linbro Chemical Co., New Haven, Conn.) (10<sup>6</sup> cells per well); after incubation at 37°C, in a 5% CO<sub>2</sub> atmosphere, the adherent macrophages were washed free from contaminating lymphocytes, and further incubated in the same conditions with the appropriate concentration of radiolabeled protein. The mean yield of adherent macrophages was 3  $\times$  10<sup>5</sup> cells per well.

*Reagents.* Human Lf was purified in an iron (Fe)-free form from milk by chromatography on carboxymethyl-Sephadex (4) (Pharmacia Fine Chemicals Inc., Piscataway, N. J.). Human transferrin (Tf) was purchased from Behring-Werke AG, Marburg/Lahn, West Germany. Horse spleen ferritin and Triton X-100 were from Sigma Chemical Co., St. Louis, Mo.

Lf and Tf were labeled with <sup>59</sup>Fe by mixing the protein with <sup>59</sup>Fe citrate in the presence of bicarbonate. The degree of iron saturation was calculated from the specific radioactivity of the metal. Labeling of ApoLf and FeLf with <sup>125</sup>I was performed by means of the chloramine T procedure. The labeled proteins were separated from excess isotope by filtration on Sephadex G 25.

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<sup>1</sup> *Abbreviations used in this paper:* Apo, devoid of iron; BME, basal medium of Eagle; FCS, fetal calf serum; Fe, iron; Lf, lactoferrin; MPM, mouse peritoneal macrophages; TCA, trichloroacetic acid; Tf, transferrin.

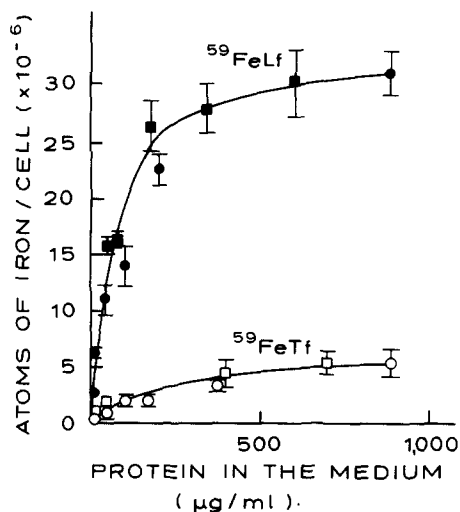


FIG. 1. Uptake of 50% saturated  $^{59}\text{FeLf}$  and  $^{59}\text{FeTf}$  by MPM after 15 h incubation as a function of the concentration of these proteins in BME containing untreated FCS (dots and circles) or transferrin-deprived FCS (open and closed squares). Vertical bars represent 1 SD. At this degree of saturation one atom of iron corresponds to one molecule of Lf or Tf.

Ultrogel AcA 34 was from LKB, Bromma, Sweden, and dextran sulfate (dextran-500) from Pharmacia Fine Chemicals.

**Methods.** After incubation with Lf or Tf, the cells were successively washed with 2 and 1 ml phosphate-buffered saline and counted. The cells were then extracted with 0.1% Triton X-100 in distilled water and the radioactivity was counted. Each result was corrected for the radioactivity recovered from the wells having been in contact with labeled proteins in the absence of cells. Two washings were sufficient to reduce the values of the blanks below 15% of the amount of protein bound by the cells.

Horse spleen ferritin was determined in fractions of gel filtration by nephelometric immunoassay by using the AIP system from Technicon Instruments Corp., Tarrytown, N. Y.

Some experiments were performed with FCS devoid of Tf. This protein was specifically removed by passing the FCS through a column of insolubilized goat anti-bovine Tf antibodies. The adsorbent was prepared by coupling the immunoglobulins from the goat anti-Tf serum to Sepharose 4B according to Cambiaso et al. (5). As shown by nephelometric immunoassay, less than 14% of the concentration of Tf present in the untreated FCS was detected in the serum passed through the immunoabsorbent column. The intracellular digestion of  $^{125}\text{I}$ -labeled proteins was followed by measuring the trichloroacetic acid (TCA)-soluble  $^{125}\text{I}$  appearing in the medium. After mixing 40  $\mu\text{l}$  of FCS as a carrier with 200  $\mu\text{l}$  of culture medium, TCA was added up to a final concentration of 10%. The amount of digested material released by the cells was calculated after correction for the TCA-soluble  $^{125}\text{I}$  present in the medium not incubated with the cells.

## Results

**Effect of the Concentration of Lf and Tf on Their Uptake by MPM.** MPM were incubated for 15 h with increasing concentrations of 50% saturated  $^{59}\text{FeLf}$  and 50% saturated  $^{59}\text{FeTf}$ . The uptake raised steeply up to a concentration of about 200  $\mu\text{g/ml}$ . Higher concentrations caused only a slight increase of the uptake (Fig. 1). For  $^{59}\text{FeTf}$ , the uptake raised slightly with concentration without clear saturation (Fig. 1). At a protein concentration of 200  $\mu\text{g/ml}$ , the amount of  $^{59}\text{Fe}$  associated with the cells was 10 times higher after incubation with Lf than with Tf.

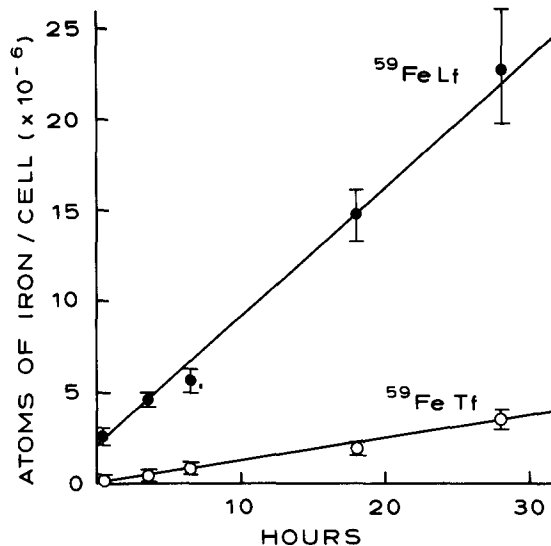


FIG. 2. Uptake of 50% saturated  $^{59}\text{FeLf}$  or  $^{59}\text{FeTf}$  by MPM as a function of time. The vertical bars represent 1 SD. The medium contained  $81 \mu\text{g}$  of Lf or  $87 \mu\text{g}$  of Tf per ml. At 50% saturation, one atom of iron corresponds to one molecule of Lf or Tf.

These experiments were performed with normal FCS and with FCS which had been deprived of its endogenous Tf by immunoadsorption. No significant difference was observed between the results of the experiment performed with the two types of culture medium (Fig. 1).

*Kinetics of FeLf and FeTf Uptake by MPM.* For both proteins, iron was found to accumulate linearly with time in the cells (Fig. 2). However, for Lf, this rate of  $^{59}\text{Fe}$  uptake was about six times faster than for FeTf. The extrapolation of the curve to zero time showed that in contrast to Tf, Lf bound to MPM instantaneously.

To estimate the amounts of FeLf present, respectively, on the cellular membrane and in the cells, the uptake of  $^{59}\text{FeLf}$  was measured after exchange of the membrane bound protein with cold FeLf. For this purpose, after incubation for various times, the  $^{59}\text{FeLf}$ -containing medium ( $40 \mu\text{g}/\text{ml}$ ) was removed, and the cells reincubated for a further 30 min with a large concentration of cold FeLf ( $10 \text{ mg}/\text{ml}$ ). The exchange between the molecules of cold FeLf and  $^{59}\text{FeLf}$  on the membrane indicated that, after 7 h, the quantity of protein bound to the membrane remained almost constant, whereas the intracellular Lf (not exchangeable) increased linearly (Fig. 3). A possible exchange of the iron between free and membrane-bound protein was discarded by experiments performed with  $^{125}\text{I}$ -labeled FeLf, which gave about the same results.

The uptake experiments were also performed by using a sample of 27% saturated Lf labeled with  $^{59}\text{Fe}$  and  $^{125}\text{I}$  (Fig. 4). Whereas the quantity of metal associated with the cells increased linearly with time, the quantity of  $^{125}\text{I}$  recovered from the cellular extracts reached a plateau after 24–30 h, suggesting that the iron was released from Lf.

*The Transfer of Iron from Lf to Ferritin in MPM.* To know the intracellular fate of iron introduced by Lf in the cells, MPM were incubated for various times

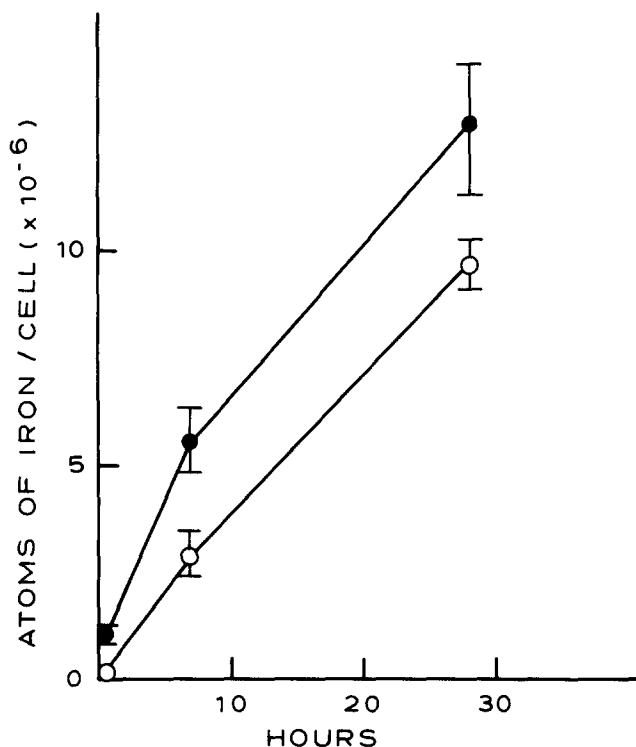


FIG. 3. Uptake of  $^{59}\text{FeLf}$  at a concentration of  $40 \mu\text{g/ml}$  by MPM as a function of time. Dots represent the total  $^{59}\text{FeLf}$  associated with the cells, whereas the circles represent the nonexchangeable iron. Vertical bars represent 1 SD.

with  $^{59}\text{FeLf}$ . Then, samples of MPM were extracted with 0.1% Triton X-100. The cell saps were fractionated on a column of Ultrogel AcA 34 in 0.1 M Tris buffer, pH 8.5, containing 1 M NaCl (Fig. 5). The radioactive iron was eluted in two major peaks. They corresponded to ferritin and Lf, respectively, as suggested by the elution diagram of horse spleen ferritin and FeLf added as markers to the cell extracts. After 6 h incubation of  $^{59}\text{FeLf}$  with MPM, 25% of the metal ingested by the cells was associated with ferritin, and 72% after 48 h.

In some fractionations of the cellular extracts obtained after 6 and 24-h incubations, a small peak was visible behind that of Lf. This retarded material corresponded to fragments of Lf as shown by experiments performed with  $^{125}\text{I}$ -labeled  $^{59}\text{FeLf}$  (Fig. 6). Both radioactivities were recovered in the main Lf peak and in the retarded peak.

*The Effect of the Concentration of ApoLf and ApoTf on Their Uptake by MPM.* The uptake of  $^{125}\text{I}$ -labeled ApoLf increased linearly with concentration up to  $200 \mu\text{g/ml}$  (Fig. 7). Beyond this concentration, the curve flattened progressively. The uptake of  $^{125}\text{I}$ -ApoTf, which increased linearly with concentration, was about 50 times lower than that of  $^{125}\text{I}$ -ApoLf for a protein concentration in the medium of  $200 \mu\text{g/ml}$ .

*Kinetics of ApoLf Uptake by MPM.* The uptake of ApoLf was compared to

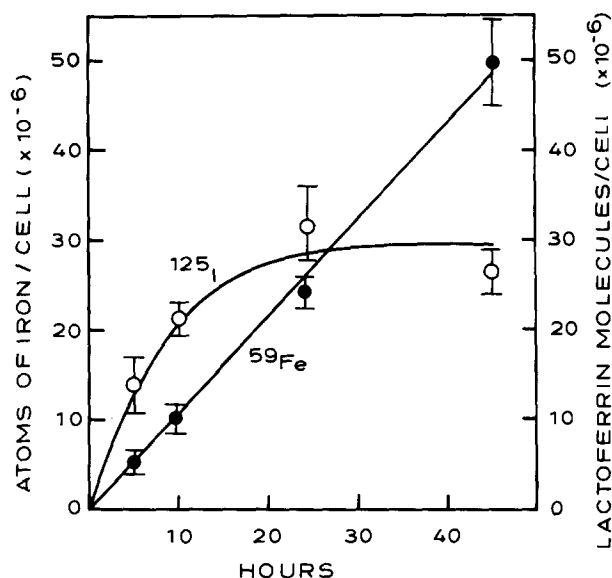


FIG. 4. Uptake of  $^{125}\text{I}$ -labeled  $^{59}\text{FeLf}$  by MPM. The medium contained 275  $\mu\text{g/ml}$  of 27% iron-saturated Lf. Vertical bars represent 1 SD.

that of FeLf, both forms of the protein being labeled with  $^{125}\text{I}$  (Fig. 8 A). After 6 h incubation, the amount of  $^{125}\text{I}$  associated with the cells was about the same for ApoLf and FeLf, but after this time a difference increasing with time appeared between the two forms of Lf. After 25 h, the extracts of cells having been incubated with FeLf contained two times more  $^{125}\text{I}$  than after incubation with ApoLf. This difference was still more evident when the experiments were carried out in the presence of 100  $\mu\text{g/ml}$  dextran sulfate (Fig. 8 B), which is known to stimulate pinocytosis (6).

The difference between the results obtained with Fe and ApoLf may be explained either by a difference in the amount of the proteins ingested, or by the rate of their elimination from the cells after a similar ingestion rate. The latter process was shown to be responsible for the lower accumulation of ApoLf in MPM by the following experiments. The cells were exposed to  $^{125}\text{I}$ -FeLf or  $^{125}\text{I}$ -ApoLf for 42 h, and the decay of radioactivity in the cells maintained in a Lf-free medium was monitored (Fig. 9). The half life of FeLf was 14.5 h, whereas that of ApoLf was 4.2 h.

This decay was not due to the release of Lf from the membrane but was really due to intracellular digestion. This was shown for FeLf by the experiments on the transfer of Fe to ferritin (see above), where fragments of Lf were recovered from the cellular extracts, and for ApoLf by measuring the appearance in the culture medium of TCA-soluble  $^{125}\text{I}$  corresponding to degradation products (Fig. 10). As a control,  $^{125}\text{I}$ -ApoLf was incubated for 30 h with the medium recovered from a 24-h MPM culture. Enough proteases were released from one cell to digest  $17 \times 10^6$  molecules of Lf. This extracellular proteolysis accounted for about one third of the total proteolysis occurring inside and outside the cells.

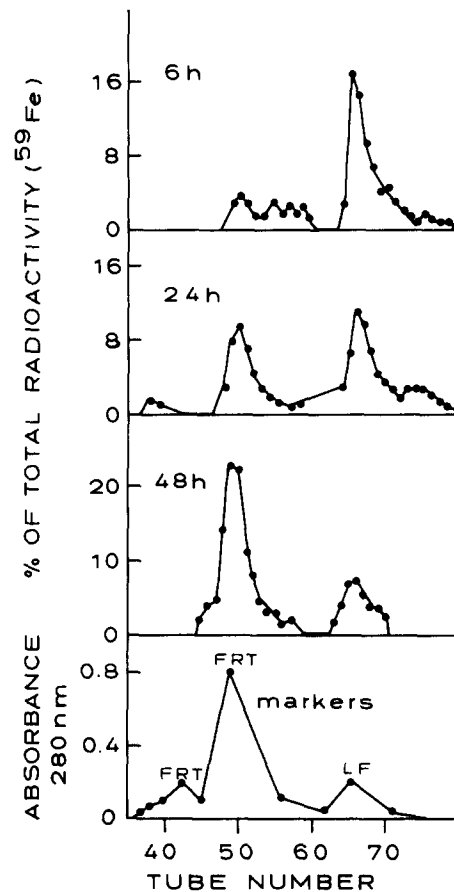


FIG. 5. Fractionation on Ultrogel AcA 34 of cellular extracts from MPM having been incubated for various times with  $^{59}\text{FeLf}$ . The column was calibrated with horse spleen ferritin (FRT) and Lf.

### Discussion

The present work shows that (a) Lf is ingested by MPM, (b) this ingestion proceeds via a membrane receptor, (c) the iron bound to Lf is transferred to ferritin after digestion of Lf, (d) ApoLf is digested in the cells faster than is FeLf.

(a) The ingestion of FeLf was shown by the recovery of Lf fragments in the cellular extracts and by the enhancing effect of dextran sulfate on the uptake of FeLf and ApoLf. The release of TCA-soluble  $^{125}\text{I}$  by MPM after incubation with  $^{125}\text{I}$ -labeled ApoLf is a further argument since the proteases released in the medium could not account for such amounts of digestion products. In most experiments with FeLf or FeLf we have labeled the proteins with  $^{59}\text{Fe}$  rather than with  $^{125}\text{I}$ , to avoid a possible alteration of the molecule by iodination. As seen earlier (3), the integrity of Lf is critical for its interaction with macrophages.

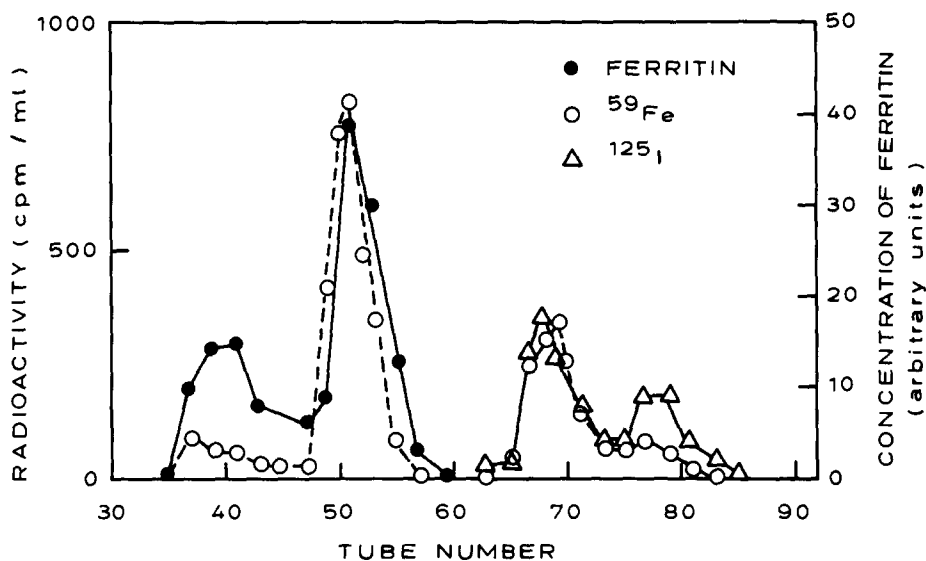


FIG. 6. Fractionation on Ultrogel AcA 34 of the cellular extracts from MPM which were incubated with <sup>125</sup>I-labeled <sup>59</sup>FeLf for 45 h. The elution of horse spleen ferritin, used as a marker, was monitored by nephelometric immunoassay.

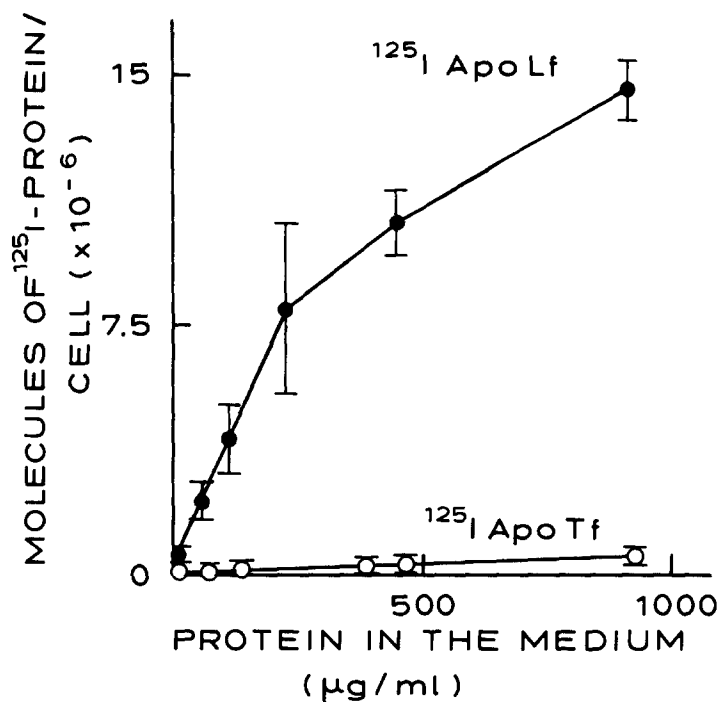


FIG. 7. Uptake of <sup>125</sup>I-ApoLf and <sup>125</sup>I-ApoTf by MPM after 15 h incubation as a function of the concentration of these proteins in the medium. Vertical bars represent 1 SD.

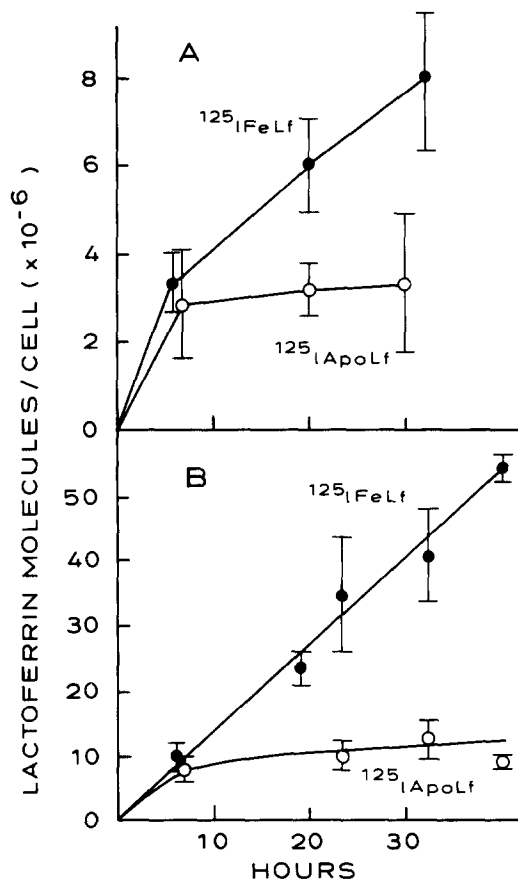


FIG. 8. Comparison of the accumulation of FeLf and ApoLf ( $100 \mu\text{g/ml}$  in the medium) by MPM, in the absence (A) or presence (B) of dextran sulfate ( $100 \mu\text{g/ml}$ ). The vertical bars represent 1 SD.

(b) In a preceding paper (3) we have shown that MPM possess a specific receptor for Lf. That Lf is ingested via this receptor rather than by fluid endocytosis is demonstrated by the study of the effect of the concentration of FeLf or ApoLf on their cellular uptake. For both forms of protein, in contrast to Tf, the uptake reached a saturation point. The latter is less evident for ApoLf because of its faster intracellular digestion leading to the release of  $^{125}\text{I}$  in the medium. This saturation concerned mainly the ingestion process since, after 15 h, which was the incubation time for the study of the effect of concentration, 67% of the Lf associated with the cells could not be exchanged with cold Lf added to the medium. The low uptake of Tf was not due to a competitive inhibition by the endogenous Tf of the FCS. The results of the experiments repeated in FCS which had been specifically deprived of its endogenous Tf did not differ from those obtained with the usual FCS.

(c) Concerning the transfer of iron from Lf to ferritin, several questions remain unanswered. From our experiments it is not possible to say whether an intermediate factor is involved in this transfer, and whether it occurs in the



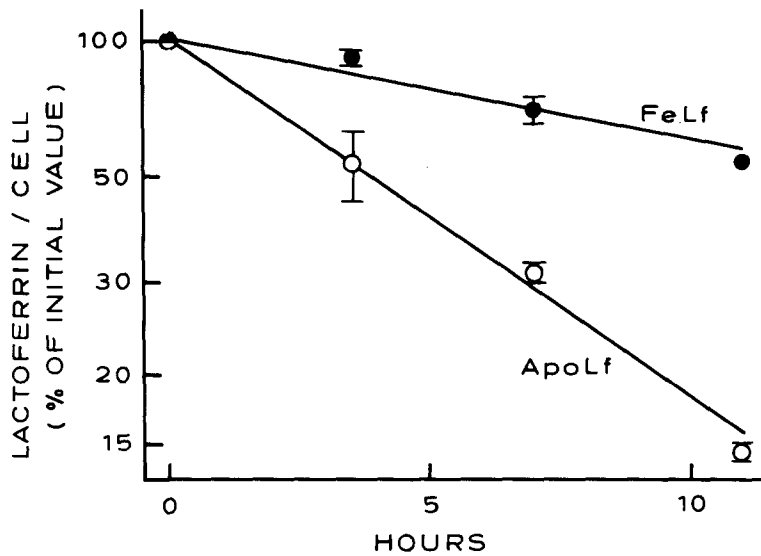


FIG. 9. Elimination of FeLf and ApoLf from MPM monitored by the decay of radioactivity associated with the cells. The cells were incubated with the proteins during 42 h, then washed twice and further incubated in medium free of Lf.

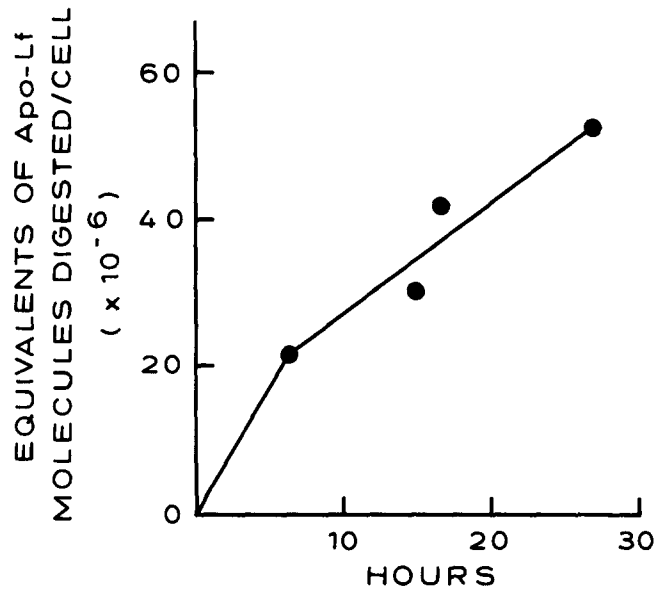


FIG. 10. TCA-soluble <sup>125</sup>I released from MPM after incubation with 100 μg/ml <sup>125</sup>I-ApoLf as a function of time.

phagolysosomes or in the cytoplasm, where the presence of ferritin has been reported (7).

Recently, half molecules of Lf capable of binding iron have been prepared by pepsin hydrolysis in mild conditions (8). So the presence of Lf fragments containing iron in the cellular extracts can be explained by partial digestion starting before the removal of iron which will be released after further proteolysis.

Owing to their similarities in structure (4) and iron-binding capacity (2), Lf is generally compared to Tf. Hence the interaction of Lf with macrophages could to some extent be studied in the light of the binding of Tf to reticulocytes, and the transfer of iron from this protein to these cells. However, the fate of Lf appears to be quite different since both Apo and FeLf are digested after ingestion by macrophages in contrast to Tf which leaves the cells in an intact metal-free form (9).

(d) It is not surprising that ApoLf is digested faster than FeLf since previous studies have already shown that the metal-saturated protein is more resistant in vitro to various proteolytic enzymes (10). The digestion of Lf in the cells might explain why the concentration of this protein in plasma has always been found to be very low, i.e. less than 1  $\mu\text{g/ml}$  even in inflammatory conditions with high counts of circulating neutrophils (11). Lf which is released by these cells is avidly picked up by macrophages and so cannot be recovered in the plasma.

It is known that the iron which is recovered from the catabolism of erythrocytes in the reticuloendothelial system passes into circulation via two pathways, a fast one with a half time of 24 min and a slow one with a half time of 7 days. Under normal conditions, the amounts of iron passing through each pathway are almost similar, whereas during inflammation, the slow pathway is more important (12). We have previously proposed that Lf absorbed onto the membrane of the macrophage, reintroduces into the cells the iron released after the destruction of erythrocytes (13). The transfer of this iron by Lf into ferritin might represent the slow pathway described in the kinetic studies.

### Summary

Human lactoferrin (Lf) labeled with  $^{125}\text{I}$  and/or  $^{59}\text{Fe}$  was found to be ingested in vitro by mouse peritoneal macrophages (MPM). The uptake measured after 15 h incubation reached a saturation point at a concentration of 200  $\mu\text{g/ml}$  in the culture medium, whatever was the iron content of Lf. In such conditions, the uptake of transferrin (Tf) used as a control was 10 times lower. At a concentration of 80  $\mu\text{g/ml}$  in the medium, one cell picked up about  $0.7 \times 10^6$  molecules of Lf per hour, and  $0.13 \times 10^6$  molecules of Tf per hour. Iron-saturated Lf disappeared from MPM with a half life of 14.5 h, whereas the half life of iron-free Lf was 4.2 h. Concomitant with the intracellular digestion of Lf, the iron was transmitted to ferritin.

These data provide additional support for the hypothesis that Lf plays a key role in iron turnover, especially at the level of the reticuloendothelial system where iron is recovered from the catabolism of erythrocytes.

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