Uptake and handling of iron from transferrin, lactoferrin and immune complexes by a macrophage cell line

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The murine macrophage-like cell line P388D1 has been used as a model to investigate whether iron acquired simultaneously from different sources (transferrin, lactoferrin, and ovotransferrin–anti-ovotransferrin immune complexes) is handled in the same way. P388D1 cells bound both lactoferrin and transferrin, but over a 6 h incubation period only the latter actually donated iron to the cells. When the cells were incubated with [⁵⁵Fe]transferrin and [⁵⁹Fe]ovotransferrin–anti-ovotransferrin immune complexes iron was acquired from both sources. However, there was a difference in the intracellular distribution of the two isotopes, proportionally more ⁵⁵Fe entering haem compounds and less entering ferritin. When the cells were precultured in a low-iron serum-free medium almost no transferrin-iron was incorporated into ferritin, whereas the proportion of immune complex-derived iron incorporated into ferritin was unchanged. Lactoferrin enhanced the rate of cellular proliferation, as measured by [³H]thymidine incorporation, despite its inability to donate iron to the cells, suggesting a stimulatory effect independent of iron donation. In contrast immune complexes inhibited cell proliferation. These findings indicate that iron acquired from transferrin and iron acquired by scavenging mechanisms are handled differently, and suggest that more than one intracellular iron transit pool may exist.

INTRODUCTION

Cellular uptake of iron may occur by a number of different routes. The most well characterized is that of uptake from transferrin, which occurs by a process of receptor-mediated endocytosis during which the cell removes iron from transferrin and releases apotransferrin to the extracellular fluid (Huebers & Finch, 1987). The expression of transferrin receptors is linked to the cell's requirement for iron, and this route of uptake is thus controlled by the level of metabolically available iron within the cell. However, many cells, particularly the mononuclear phagocytes of the liver and spleen, also acquire iron as a result of erythrophagocytosis during the normal process of removal of effete red cells. Iron is released from haemoglobin by haem oxygenase and is then either released from the cell or incorporated into ferritin (Lynch *et al.*, 1974). In inflammatory lesions, macrophages may also phagocytose other iron-containing cellular debris, and it has been proposed that they may also acquire iron from lactoferrin released by degranulating neutrophils (Van Snick *et al.*, 1977). Much less is known about the intracellular handling of iron acquired via these essentially scavenging mechanisms, but it seems unlikely that uptake is controlled by intracellular iron levels in the same way as uptake of transferrin-bound iron.

It is currently believed that iron entering a cell passes through an intracellular labile or metabolic pool, from which it may either be used for cellular metabolism, incorporated into ferritin, or released from the cell (Jacobs, 1977). However, iron in excess of that which can be bound by ferritin, transferrin or other macromolecules

is potentially toxic, and it thus seems possible that iron acquired by scavenging routes might be handled differently from iron acquired from transferrin, as the former needs to be disposed of whereas the latter needs to be utilized. The fact that cells can take up iron from the chelator nitrilotriacetate and incorporate the metal into ferritin (White & Jacobs, 1978; Brock & Rankin, 1981; Alvarez-Hernández *et al.*, 1986), yet cannot utilize this form of iron to permit cellular proliferation (Brock, 1981; Landschulz & Ekblom, 1985), also argues for more than one route of intracellular iron handling.

One approach to investigating this question would be to study iron handling by a cell which is capable of simultaneously acquiring iron from transferrin and by a scavenging route. To accomplish this we have examined uptake and intracellular distribution of iron in the murine macrophage cell line P388D1. This cell line expresses receptors for Fc and C3b (Koren *et al.*, 1975) and also acquires iron from transferrin (I. Esparza & J. H. Brock, unpublished work). We have previously shown that macrophages readily take up iron from transferrin–anti-transferrin immune complexes, presumably via their Fc receptors, and that this iron may be either released or incorporated into ferritin (Esparza & Brock, 1981; Brock *et al.*, 1984; Alvarez-Hernández *et al.*, 1986). From the work of Van Snick *et al.* (1977) one might also expect this macrophage cell line to interact with lactoferrin. We have therefore investigated the ability of P388D1 cells to acquire iron from transferrin, lactoferrin, or immune complexes, and studied the subsequent intracellular distribution of the metal. We have also examined the ability of these three potential iron donors to promote cell proliferation.

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EXPERIMENTAL

Proteins and sera

Human apotransferrin and albumin were obtained from Behringwerke (Hoechst, Hounslow, U.K.). Ovontransferrin (30% iron-saturated) was kindly provided by Dr R. W. Evans (Guy's Hospital, London, U.K.). Human lactoferrin was prepared from colostrum by the method of Johansson (1969) and bovine lactoferrin from dry-period udder secretion according to the method of Reiter *et al.* (1975). Lactoferrins were rendered iron-free by dialysis against EDTA-citrate at pH 4 as described by Mazurier & Spik (1980). Ferritin was prepared from mouse spleen by the method of Linder & Munro (1972). Anti-sera to ovotransferrin, mouse spleen ferritin and human transferrin were raised in rabbits.

Cell culture

P388D1 cells were kindly provided by Dr M. Turner (Department of Zoology, Glasgow University, Glasgow, U.K.). They were routinely cultured in medium consisting of Roswell Park Memorial Institute medium 1640 (RPMI 1640; Flow Laboratories, Rickmansworth, U.K.) supplemented with 10% fetal-calf serum (Flow), penicillin (100 units/ml; Flow), streptomycin (100 µg/ml; Flow), fungizone (2.5 µg/ml; Sigma, Poole, Dorset, U.K.) and gentamycin (50 µg/ml; Sigma). The cells were adapted to growth in a serum-free medium consisting of RPMI 1640 plus human serum albumin (1 mg/ml), apotransferrin (50 µg/ml) and the antibiotics by progressively diluting out the fetal-calf serum at sub-culture, which was performed every 2–3 days. Cells were maintained in this medium for several weeks. All interactions of cells with iron-binding proteins were carried out at 37 °C.

Radiolabelling of proteins

Transferrins were labelled by adding sufficient ⁵⁹Fe-citrate (Amersham; specific activity 10 µCi/µg) or ⁵⁵FeSO₄ (Amersham; specific activity 0.79 mCi/µg) to saturate the proteins, which were dissolved in phosphate-buffered saline containing 1% NaHCO₃ and left overnight to ensure specific binding of iron. If required, they were then labelled with ¹²⁵I (500 µCi/mg of protein) by the chloramine-T method (Greenwood *et al.*, 1963), the reaction time being limited to 1 min to avoid denaturation. Unbound radioactive Fe or ¹²⁵I were removed by passage through a column of Chelex-100 resin (Bio-rad, Gloucester, U.K.) or Sephadex G25 (Pharmacia, Uppsala, Sweden) respectively. Lactoferrins showed a tendency to bind to Chelex resin and in this case unbound Fe was removed by adding resin to the protein solutions, shaking gently for 1 min and then removing the liquid with a Pasteur pipette. This was found to remove all unbound iron while causing only minimal losses of lactoferrin.

Preparation of immune complexes

Soluble immune complexes were formed by rapidly mixing 5 vol. of ovotransferrin (0.1 mg/ml) with 1 vol. of anti-ovotransferrin serum. The reaction was carried out in the presence of complement, which prevents precipitation of the resulting immune complexes (Schifferli *et al.*, 1980). To ensure the presence of complement, the anti-ovotransferrin serum was frozen at -70 °C in small portions immediately after separation from the clot, and

each portion used only once. The complexes were centrifuged for 10 min in a microcentrifuge at 10000 g to remove any traces of insoluble complexes and used immediately.

Interaction of P388D1 cells with ¹²⁵I-[⁵⁹Fe]lactoferrin and transferrin

Logarithmic-phase P388D1 cells in serum-containing medium were centrifuged and resuspended in four tubes each containing 2.5 ml of medium (2 ml of fresh medium plus 0.5 ml of original growth medium). The cell concentration was (5–10) × 10⁶/ml. To each of two tubes was added ¹²⁵I-[⁵⁹Fe]transferrin and unlabelled lactoferrin (5 µg/ml in each case) while labelled lactoferrin and unlabelled transferrin was added to the other two. After 6 h incubation the cells were centrifuged for 1.5 min at 200 g and washed twice in ice-cold phosphate-buffered saline. Before the final centrifugation the suspensions were transferred to fresh tubes to minimize errors due to binding of labelled proteins to the tubes. The ⁵⁹Fe and ¹²⁵I activity in the supernatants, washes and cell pellets, were counted for 5 min in a LKB Compugamma counter (LKB, Croydon, U.K.) and uptake of each isotope was calculated.

Uptake of iron from transferrin and ovotransferrin-anti-ovotransferrin immune complexes

Cell suspensions (10 ml, 2 × 10⁶ cells/ml) were prepared in duplicate in tissue culture flasks as described above, using cells grown in either serum-containing or serum-free media. Each flask received [⁵⁹Fe]transferrin (50 µg/ml) and [⁵⁹Fe]ovotransferrin-anti-ovotransferrin immune complexes (50 µg/ml of ovotransferrin) and was then incubated overnight (15 h). The cells were then washed twice in phosphate-buffered saline and subjected to a previously described fractionation procedure (Alvarez-Hernández *et al.*, 1986). Briefly, this consisted of lysing the cells in phosphate-buffered saline/1% Triton X-100 containing 1 mM-desferrioxamine (CIBA-Geigy, Horsham, U.K.), centrifugation at 10000 g for 5 min, affinity chromatography on Sepharose-anti-ferritin and ultrafiltration through a 10000 molecular mass cut-off filter. This allows separation of intracellular iron into four compartments, i.e. insoluble (haemosiderin-like) iron, ferritin iron, low-molecular-mass chelatable iron and high-molecular-mass non-ferritin iron. Haem iron was estimated by acid cyclohexanone extraction (Esparza & Brock, 1981) of the retained fraction from the ultrafiltration step.

All samples were counted for ⁵⁵Fe and ⁵⁹Fe in a Packard 4450 scintillation counter programmed to correct for spill-over of ⁵⁹Fe into the ⁵⁵Fe channel.

Measurement of cell proliferation

Cells which had previously been maintained in serum-free medium were sub-cultured at 6 × 10⁵/ml into 2 ml of fresh medium plus 1 ml of conditioned serum-free medium and lactoferrin, transferrin or immune complexes added (50 µg/ml of iron-binding protein in each case). Control cultures were set up containing no additions (though all cultures would have contained 17 µg of apotransferrin/ml carried through in the conditioned medium; it was not possible to culture cells in serum-free transferrin-free medium). Each culture received 100 µCi of [³H]thymidine/ml (Amersham; specific activity 5 Ci/mmol). At 6 h and 24 h, 1.2 ml was

removed from each culture and split into $12 \times 100 \mu\text{l}$ aliquots on a microtitre plate. The cells were harvested and thymidine incorporation measured by scintillation counting.

RESULTS

Interaction of lactoferrin and transferrin with P388D1 cells

P388D1 cells bound significant amounts of both lactoferrin and transferrin (Table 1), 3–4 times as much lactoferrin being bound as transferrin. However, the ratio of ^{59}Fe to ^{125}I associated with the cells clearly indicated that whilst the cells were actively acquiring iron from transferrin, there was no net accumulation of iron from lactoferrin, all the cell-associated ^{59}Fe activity being accountable by binding of Fe_2 -lactoferrin to the cells. Both bovine and human lactoferrin behaved similarly. Thus whilst this cell line appears to acquire transferrin-bound iron, as do many other proliferating cells, it is unable to take up measurable amounts of iron

from lactoferrin, despite the ability of the protein to bind to the cells.

Uptake of iron from transferrin and ovotransferrin–anti-ovotransferrin immune complexes

The cells acquired approx. twice as much iron from transferrin as from the immune complexes (Table 2). This was true whether cells were incubated in serum-free or serum-containing medium, although overall uptake was about 3 times greater in serum-free medium, perhaps because the presence of bovine transferrin and complement partially inhibited the binding of transferrin and immune complexes to transferrin receptors and C3b receptors respectively. Fractionation of intracellular iron revealed that proportionally less transferrin-iron entered ferritin than iron derived from immune complexes. Indeed, virtually no transferrin-derived iron was incorporated into ferritin by cells previously cultured in the serum-free medium. Most of the iron acquired from transferrin was present in a soluble non-ferritin high-molecular-mass fraction, as also was the majority of the non-ferritin iron acquired from immune complexes. The low-molecular-mass fraction was about one-third the size of the non-ferritin high-molecular-mass fraction, irrespective of iron donor or culture medium. Only small amounts of iron were present in insoluble form.

In separate experiments, the haem content of the non-ferritin high-molecular-mass fraction was determined. It was found that $62 \pm 11\%$ of the transferrin-derived iron in this fraction was extractable by acid/cyclohexanone, as compared with $22 \pm 8\%$ of the iron acquired from immune complexes (means $\pm$ S.D.; $n = 3$).

Effect of different forms of iron on cell proliferation

During the first 6 h cells cultured in the presence of lactoferrin incorporated more thymidine than the other cultures (Table 3). By 24 h incorporation into the cultures containing immune complexes was clearly much lower than into the other cells, whereas the control and transferrin-containing cultures had by now reached a level of incorporation similar to that of the lactoferrin-containing cultures. Thus lactoferrin appears to enhance

Table 1. Interaction of ^{125}I - ^{59}Fe lactoferrin and transferrin with P388D1 cells

P388D1 cells at $(5\text{--}10) \times 10^6/\text{ml}$ were incubated for 6 h with doubly labelled transferrin and unlabelled lactoferrin, or *vice versa*. Each protein was added at 65 pmol/ml. Results are means of duplicate cultures which did not differ from the means by more than 8%.

	^{125}I -protein bound (pmol/ 10^6 cells)	^{59}Fe uptake (pmol/ 10^6 cells)	Iron/ protein molar ratio
Human transferrin	0.052	1.113	21.4
Bovine lactoferrin	0.217	0.443	2.0
Human lactoferrin	0.141	0.204	1.5

Table 2. Distribution of iron in P388D1 cells following uptake from transferrin and immune complexes

P388D1 cells at $2 \times 10^6/\text{ml}$ were incubated with ^{59}Fe transferrin and ^{59}Fe ovotransferrin–anti-ovotransferrin immune complexes (65 pmol of iron-binding protein/ml in each case) for 15 h. The medium was RPMI 1640 supplemented with either 10% fetal-calf serum or 50 μg of apotransferrin/ml and 1 mg of human serum albumin/ml. Fractionation of intracellular iron was performed as described in the text. Figures are means of duplicate cultures which did not differ from the means by more than 15%.

Medium . . .	Iron uptake (pmol/ 10^6 cells)			
	Serum-containing		Serum-free	
	Transferrin	Immune complexes	Transferrin	Immune complexes
	0.38	0.20	1.32	0.61
Source of iron	Iron uptake (%)			
Ferritin	17.7	42.1	2.8	36.2
High-molecular-mass non-ferritin	59.3	43.7	70.2	43.4
Low molecular mass	20.3	11.7	25.4	13.5
Insoluble	2.7	2.5	1.6	6.9

Table 3. Incorporation of [³H]thymidine into P388D1 cells

Cells at 6×10^5 /ml were incubated in serum-free medium (2 ml fresh transferrin-free medium plus 1 ml of conditioned medium containing 50 μ g of apotransferrin/ml). All cultures therefore contained 17 μ g of (apo)transferrin/ml. Iron-binding proteins were added at 50 μ g/ml, and the cultures were pulsed with [³H]thymidine as described in the text. Values are means $\pm$ S.D. of 12 replicate wells from a single representative experiment.

Medium	Incubation time (h)...	10 ⁻³ $\times$ [³ H]Thymidine (c.p.m./10 ⁶ cells)	
		6	24
Control (no addition)		72 $\pm$ 31	203 $\pm$ 60
+ Lactoferrin		163 $\pm$ 41	213 $\pm$ 38
+ Transferrin		70 $\pm$ 13	267 $\pm$ 80
+ Immune complexes		59 $\pm$ 18	98 $\pm$ 37

cell proliferation, whilst ovotransferrin-anti-ovotransferrin had the opposite effect. The lack of differences between the control and transferrin-containing cultures can be attributed to the fact that transferrin was carried over into all cultures from the conditioned medium.

DISCUSSION

The binding of lactoferrin to macrophages was first reported by Van Snick & Masson (1976), who suggested that specific lactoferrin receptors were involved. This finding has subsequently been confirmed by others (Birgens *et al.*, 1983) and the binding of lactoferrin to adenocarcinoma cells (Amouric *et al.*, 1984) and lymphocytic cell lines (Hashizume *et al.*, 1983) has also been reported. However, the ability of lactoferrin to donate iron to cultured cells appears to have been investigated only by Van Snick *et al.* (1977), who reported that lactoferrin bound by macrophages was degraded, and the iron incorporated into ferritin. Whilst the ability of P388D1 cells to bind lactoferrin, reported here, is in line with previous observations, we were unable to detect any significant uptake of iron from the carrier protein by the cells. However, the results are not as divergent from those of Van Snick *et al.* (1977) as they might at first appear, because the rate of iron uptake detected by these workers was extremely slow, with detectable net accumulation of iron and incorporation into ferritin only being observed after about 24 h of incubation. These results do not rule out the possibility that lactoferrin may enter cells by some kind of receptor-mediated process analogous to the uptake of transferrin but if it does occur it would seem that lactoferrin does not readily donate iron to the cell in the process. Such a mechanism actually occurs during the transit of lactoferrin through hepatic cells (Regoezi *et al.*, 1985). It should be emphasized that both lactoferrin and transferrin were present in all cultures, and that lack of acquisition of lactoferrin-bound iron cannot therefore be due to metabolic differences between the two cultures.

Interaction of lactoferrin with cell lines has been reported to enhance the rate of cellular proliferation (Amouric *et al.*, 1984; Hashizume *et al.*, 1983). This was also found to be the case for P388D1. However, the

negligible iron uptake from lactoferrin, and the ability of lactoferrin to enhance growth even in the presence of transferrin, from which iron is much more rapidly acquired, indicates that the growth-promoting effect of lactoferrin cannot be due to an iron-donating role. Nevertheless, lactoferrin clearly has an effect on cell proliferation, and the mechanism involved deserves further study.

It was originally intended to use lactoferrin as an alternative source of iron to transferrin for studies of intracellular distribution. In view of the lack of uptake of lactoferrin-bound iron, an alternative source was chosen, namely ovotransferrin-anti-ovotransferrin immune complexes. We have shown previously that macrophages readily take up such immune complexes and that this forms a useful model for investigating the handling of iron acquired by a scavenging type of mechanism (Esparza & Brock, 1981; Brock *et al.*, 1984; Alvarez-Hernández *et al.*, 1986). Ovotransferrin was used in preference to human or other mammalian transferrins as it is not bound by mammalian transferrin receptors (Zapolski & Princiotta, 1976; Shimo-Oka *et al.*, 1986) and thus the immune complexes would be unlikely to enter by the transferrin receptor-mediated route. In addition, the large insoluble complexes used in previous studies are degraded relatively slowly (Esparza & Brock, 1981), so for the present study soluble complexes were used, taking advantage of the fact that immune complexes remain soluble if formed in the presence of complement (Schifferli *et al.*, 1980).

The results reported here show that when P388D1 cells acquire iron simultaneously from transferrin and immune complexes, the metal from these two different sources is not handled identically, proportionally more of the iron from immune complexes being routed to ferritin, and less to haem compounds. This strongly suggests that iron acquired from transferrin is preferentially routed to metabolic processes, while iron acquired simultaneously by a scavenging route tends to be stored. Only when cells were precultured in medium containing fetal-calf serum did appreciable amounts of transferrin-iron reach ferritin. The transferrin in fetal-calf serum is generally about 50% saturated with iron (Brock *et al.*, 1986), and it might be argued that cells grown in such media are exposed to an unphysiologically iron-rich environment. A considerable amount of iron acquired from both sources was present in a form which, though of high molecular mass, consisted of neither ferritin nor haem compounds, and the nature of this fraction clearly requires investigation. The amount of iron in cell lysates which could be chelated by desferrioxamine was always about one-third of the amount present in the non-ferritin high-molecular-mass pool.

The implications of these findings are that the idea of a single transit or labile intracellular iron pool (Jacobs, 1977) may not be correct. Iron is released from transferrin in an endosome (Huebers & Finch, 1987) whereas iron taken up as immune complexes will be released in a phagolysosome. Possibly these two different intracellular compartments release their iron to different transit pools, that from transferrin being mainly directed towards cellular metabolism and that from lysosomes towards iron storage (and perhaps release). Studies to test this hypothesis are under way. Evidently there must be some degree of interchange between these two proposed transit pools, as some transferrin-iron was found in ferritin

when the cells were cultured in iron-rich conditions, and some immune complex-derived iron was incorporated into haem. Nevertheless, the existence of two transit pools with only a limited degree of interchangeability could explain why iron taken up from non-transferrin sources (e.g. nitrilotriacetate or citrate complexes) is frequently unable to support cell proliferation (Brock 1981; Landschulz & Ekblom, 1985).

It should be pointed out that P388D1 cells produce Interleukin 1 (IL 1) (Mizel & Mizel, 1981) which is reported to depress serum iron (Kampschmidt & Mesecher, 1985), probably by altering iron handling by reticuloendothelial cells. Although release of IL 1 by P388D1 requires stimulation by agents such as phorbol esters and actinomycin D (Mizel & Mizel, 1981) the possibility remains that a small amount of IL 1 could be released and modulate intracellular iron metabolism. Further work would be necessary to determine whether this is the case.

The inhibitory effect of ovotransferrin-anti-ovotransferrin immune complexes on cell proliferation even in the presence of transferrin was unexpected. Whether it is due to a toxic effect of scavenged iron on cells which are not well-equipped to deal with such iron (for example, because of inadequate intracellular ferritin levels), or is a phenomenon unrelated to iron but modulated through Fc and/or C3b receptors, should now be investigated.

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