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Multivalent Metal-Induced Iron Acquisition from Transferrin and Lactoferrin by Myeloid Cells

Oyebode Olakanmi,*‡ George T. Rasmussen,*‡ Troy S. Lewis,* John B. Stokes,*‡ John D. Kemp,¶§ and Bradley E. Britigan‡¶†

We previously described a unique, high-capacity, ATP-independent mechanism through which myeloid cells acquire Fe from low-m.w. chelates. The rate of this Fe acquisition is markedly increased by cellular exposure to multivalent metal cations. Because most Fe in vivo is bound to transferrin or lactoferrin, we examined whether this mechanism also contributes to myeloid cell acquisition of Fe from transferrin and/or lactoferrin. Using HL-60 cells as a model system, we show cellular acquisition of $^{59}$Fe from both lactoferrin and transferrin that was unaffected by conditions that depleted the cells of ATP or disrupted their cytoskeleton. Fe acquisition was dramatically increased by cell exposure to various metals including Ga$^{3+}$, Ge$^{4+}$, Al$^{3+}$, Fe$^{3+}$, La$^{3+}$, Zr$^{4+}$, Sn$^{4+}$, Cu$^{2+}$, and Zn$^{2+}$ by a process that was reversible. Exposure to these same metals also increased binding of both transferrin and lactoferrin to the cell surface by a process that does not appear to involve the well-described plasma membrane receptor for transferrin. Approximately 60% of the Fe acquired by the cells from transferrin and lactoferrin remained cell associated 18 h later. HL-60 cells possess a high-capacity multivalent metal-inducible mechanism for Fe acquisition from transferrin and lactoferrin that bears many similarities to the process previously described that allows these and other cell types to acquire Fe from low-m.w. Fe chelates. The biologic importance of this mechanism may relate to its high Fe acquisition capacity and the speed with which it is able to rapidly adapt to the level of extracellular Fe. The Journal of Immunology, 2002, 169: 2076–2084.

Iron (Fe) is essential for the growth and metabolism of human cells. However, through its ability to promote the formation of highly reactive oxidant species it is also cytotoxic (1). Presumably for this reason and to limit its availability to pathogenic microorganisms, Fe acquisition, transport, and storage are tightly regulated. In vivo, free extracellular Fe is kept to a minimum (2). The vast majority of extracellular Fe is chelated to one of two related proteins, transferrin and lactoferrin. Transferrin serves as the principal chelator of extracellular Fe in serum, whereas lactoferrin and transferrin both serve this function at mucosal surfaces (3, 4). In addition, extracellular Fe is also bound to a variety of low-m.w. organic chelating agents (4). The amount of these low-m.w. Fe complexes increases in some pathologic states and under conditions of relative Fe overload (5–8).

Transferrin serves an important role in intercellular transport of Fe. Receptor-mediated endocytosis via a transferrin-specific plasma membrane receptor has been intensely studied and is felt to be the principal mechanism through which most cells acquire Fe from the extracellular environment (9–11). The transferrin receptor has greater affinity at physiologic pH for diferric transferrin than the apo form of the protein. Transferrin binding is followed by internalization of the receptor-transferrin-Fe complex via endocytosis (9, 10). The Fe is released, in part, by acidification of the endosome and reduction of Fe$^{3+}$ to Fe$^{2+}$ (9, 10). The Fe$^{2+}$ is then transported to the cytoplasm through the action of the divalent metals transporter 1 (DMT-1), also known as Nramp2 and DCT-1 (12). The transferrin receptor is then recycled to the plasma membrane, where apotransferrin is released from the receptor (9, 10). The efficiency of Fe acquisition from transferrin is influenced by the association of other proteins, HFE and $\beta_2$-microglobulin, with the transferrin receptor (13–15). The absence of functional HFE appears to play a critical role in hereditary hemochromatosis (16).

Other mechanisms may be involved in Fe acquisition from transferrin by some cell types (17–24). In most studies the mechanism responsible remains ill defined. However, a pathway involving the intracellular protein mobilferrin in conjunction with membrane integrins has been described (21, 22). Melanotransferrin, a plasma membrane-associated Fe-binding protein in human melanoma and other cell lines, has also been implicated in some forms of Fe acquisition from transferrin (23, 24). These processes do not appear to require transferrin internalization. In addition, a second transferrin-specific plasma membrane receptor (transferrin receptor 2) has recently been described in some cell types (25–27).

The ability of cells to acquire Fe from lactoferrin and the mechanism(s) responsible have been more controversial. Lactoferrin does not bind to the transferrin receptor (28–31). It does bind to the surface of many cell types, but whether this occurs via a lactoferrin-specific receptor remains unclear (28, 30, 32–40). Even when lactoferrin binding to cells occurs, resulting Fe acquisition has not been uniformly observed (37, 38, 41, 42).

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Many cell types have been shown to acquire Fe bound to a variety of low-m.w. chelating agents in vitro (9, 12, 43–47). Several mechanisms have been proposed for this acquisition. In some cell types these processes have been linked to stimulator of Fe transport-1 (47) and DMT-1 (12, 48). DMT-1 is particularly important in the ability of intestinal epithelial cells to acquire and transport Fe from the intestinal lumen into the body (12, 49). In studies of the acquisition of Fe from low-m.w. chelates by monocytes, macrophages, neutrophils, and human myeloid cell lines we discovered that these cells possess a unique Fe uptake mechanism that functions even in the presence of marked cellular ATP depletion. Furthermore, the rate of Fe acquisition is markedly increased by prior exposure of the cells to multivalent metal cations, including Fe (44, 50). This process does not appear to require the internalization of the chelating agent. It allows for rapid acquisition of large amounts of Fe by cells, far greater than that usually associated with receptor-mediated endocytosis of Fe from transferrin (44).

Because both transferrin and lactoferrin become associated with the cellular membrane of myeloid cells (28, 32–37, 51, 52), we hypothesized that the mechanism of Fe acquisition we have described from low-m.w. chelates may also contribute to the ability of myeloid cells to acquire Fe from transferrin or lactoferrin.

**Materials and Methods**

**Preparation of iron chelates**

Ferric nitritolactate (NTA) was prepared by combining equimolar amounts of $[^{59}\text{Fe}]{\text{Cl}_3}$ (100 μCi/ml; Amersham, Arlington Heights, IL) and NTA. Diferric transferrin ($[^{59}\text{Fe}]_{2}\text{transferrin}$) and diferric lactoferrin ($[^{59}\text{Fe}]_{2}\text{lactoferrin}$) were prepared by adding $[^{59}\text{Fe}]\text{NTA}$ or $[^{59}\text{Fe}]{\text{Cl}_3}$ to human apotransferrin or apolactoferrin (Sigma-Aldrich, St. Louis, MO) at a 2:1 molar ratio, in the presence of bicarbonate (53). The mixture was incubated at 4°C overnight after which it was centrifuged and washed three times in a centriprep-30 (Amicon, Beverly, MA) to remove any $[^{59}\text{Fe}]$ that was not tightly bound to transferrin or lactoferrin.

**Cell culture and cell preparation**

The human promyelocytic HL-60 cell line was cultured in RPMI 1640 (University of Iowa Cancer Center, Iowa City, IA) supplemented with 10% FCS, glutamine (2 mM; Sigma-Aldrich), penicillin (100 U/ml), and streptomycin (100 μg/ml). Before an experiment, the cells were washed three times in HBSS and resuspended in HBSS at $5 \times 10^5$/ml and $100 \mu$l was placed in the wells of a 96-well plate. The plate was equilibrated in the incubator (5% CO$_2$ at 37°C) for 15 min. Where desired, Ga(NO$_3$)$_3$ was added at a final concentration of 1 mM and incubated for 30 min at 37°C followed by measurements of Fe acquisition over time. In some cases the cells were washed to remove extracellular Ga before measurement of Fe acquisition. All experiments were conducted in duplicate. In each experiment control wells were included which contained cells that had only been exposed to HBSS.

**Quantitation of cellular iron acquisition**

Cell suspensions were placed at 37°C for 15 min. $[^{59}\text{Fe}]$ chelate (750 nM) was then added and the cells were incubated for the desired time periods. The cell suspension was then centrifuged at 500 g for 5 min at 4°C and the medium was carefully removed. The cell pellet in each well was washed three times in the same volume of HBSS and the amount of cell-associated $[^{59}\text{Fe}]$ was determined by a gamma counter. Parallel experiments were performed each day in the absence of cells to control for possible $[^{59}\text{Fe}]$ binding to the plate or formation of non-cell-associated Fe aggregates that might have co sedimented with the cells. These values, usually between 0.05 and 0.2% of total cpm added, were subtracted from corresponding experimental samples for each time interval.

We also used a second method to separate cell-associated Fe from free Fe chelates. This method was used to ensure that Fe complexes of transferrin or lactoferrin were not simply cosedimenting nonspecifically with the cells. Aliquots of the cell suspension that had been incubated with Fe were layered on top of an oil cushion (0.5 ml, 4:1 dibutylphthalat:dimonyl) in 1.5-ml conical cryotubes and centrifuged at 10,000 × g for 10 min. The tube was then placed in a dry ice-acetone bath until the liquid solidified. Cell pellets were cut from the bottom of the tubes and the amount of cell-associated $[^{59}\text{Fe}]$ was determined using a gamma counter. Both methods used to separate cell-associated Fe from extracellular $[^{59}\text{Fe}]$ yielded similar results.

In some experiments cells were incubated with a combination of metabolic inhibitors, NaCN (1 mM) and 2-deoxy-d-glucose (50 mM), for 2 h at 37°C. After an additional 15 min of incubation at 37°C, Fe acquisition was measured as above.

**Measurement of transferrin/lactoferrin binding by HL-60**

APO- or Fe-loaded transferrin or lactoferrin were iodinated using the iodogen method (Pierce, Rockford, IL). Successful iodination of the proteins and the lack of protein degradation following the procedure were confirmed by SDS-PAGE followed by autoradiography. It revealed a single band of radioactivity for each protein migrating at ~80 kDa. More than 90–95% of $[^{125}\text{I}]$ activity resulting from placement of these iodination proteins in solution could be immunoprecipitated using polyclonal antiserum specific to that specific protein (anti-transferrin and anti-lactoferrin).

In preparation for the determination of lactoferrin or transferrin binding, Ga-treated or control cells were washed in HBSS. Cells were equilibrated at 4°C for 10 min and then incubated (2 × 10$^6$/ml) with apo- or holo-$[^{125}\text{I}]$transferrin/lactoferrin (563 nM) in a volume of 0.5 ml at 4°C for 15 min, and washed three times. Cell-associated $[^{125}\text{I}]$ was then assayed by counting the cell pellet in a gamma counter. It has previously been reported that the binding properties of lactoferrin can be altered by iodination procedures (54). However, the iodination procedure used in our work had a negligible effect on binding of either protein to the HL-60 cell surface, as demonstrated by the following results. Binding to HL-60 cells of $[^{59}\text{Fe}]$transferrin and $[^{59}\text{Fe}]$lactoferrin that had been iodinated (using nonradioactive iodine) according to our iodination protocol was compared with noniodinated controls. There was no evidence that the iodination procedure altered binding of either transferrin or lactoferrin to these cells.

**Immunoprecipitation of transferrin and lactoferrin**

Cells were lysed in TBS containing 1% Triton X-100, 0.1 mg/ml leupeptin and pepstatin A, and 2 mM PMSF (30 min at 4°C). The lysate was incubated with normal rabbit serum for 30 min followed by an additional 30 min in the presence of formalin-fixed protein A-bearing Staphylococcus aureus (Pansorbin cells; Calbiochem, San Diego, CA). The mixture was then centrifuged in a refrigerated microfuge and the supernatant was transferred to a fresh Eppendorf tube. These precleared supernatants were then incubated with polyclonal Ab to human transferrin or lactoferrin (Calbiochem) in 50 mM Tris-Cl and 190 mM NaCl containing 2.5% Triton X-100 for 4 h at 4°C. At that time protein A-Sepharose CL-4B (Amersham Pharmacia Biotech, Piscataway, NJ) was added and the incubation was continued for an additional 60 min. The immune complexes were then pelleted and washed, following which the pellet and supernatant were transferred to a gamma counter for determination of $[^{59}\text{Fe}}$ or $[^{125}\text{I}]$.

**FACS analysis**

Cells that were pre-exposed to Ga or control cells (without Ga exposure) were incubated with anti-transferrin receptor mAb (A27.15, E2.3) (55, 56), anti-MPO mAb (MPO-7), or normal mouse IgG for nonspecific staining and in the presence of FITC goat F(ab')2 anti-mouse IgG. Samples were analyzed by FACSscan flow cytometer.

**Statistical analyses**

Results obtained under different experimental conditions were compared by Student’s paired t test when independent variables were being assessed or by ANOVA when analyses of trends were being determined. For both types of analyses results were considered significant at p ≤ 0.05.

**Results**

Cationic metals enhance iron acquisition from transferrin and lactoferrin

Previous work from our laboratory indicates that mononuclear and polymorphonuclear phagocytes, as well as human myeloid cell lines such as the promyelocytic HL-60 cell line, can acquire Fe from low-m.w. chelates via a unique mechanism that does not involve receptor-mediated endocytosis (44, 50). This process is not altered by depletion of cellular ATP and the rate and magnitude of Fe acquisition is dramatically increased by cellular exposure to Fe or other polyvalent cationic metals (44, 50). In the present study we examined to what extent this mechanism may also contribute to Fe acquisition from
transferrin and lactoferrin by myeloid cells. HL-60 cells were incubated with [55Fe2+]transferrin or [55Fe2+]lactoferrin for defined time periods, at which time 55Fe associated with the cell was measured. An increase in cell-associated 55Fe was observed over time using either transferrin or lactoferrin (Fig. 1A). At each time point, the magnitude of Fe acquired was ~3-fold greater with Fe-lactoferrin relative to Fe-transferrin (Fig. 1A).

Analogous to its effect on Fe acquisition from low-m.w. chelates (44, 50), Ga(NO3)3 markedly augmented Fe acquisition by HL-60 cells from transferrin and lactoferrin (Fig. 1B). The majority of the iron acquired by the cells occurred in the first 10 min (Fig. 1B). Other trivalent (Al, Fe, Gd, La) or tetravalent (Sn, Zr) metals had a similar effect (Table I). Cell acquisition of Fe from transferrin following incubation with these multivalent ions was increased 5- to 18-fold. The fold stimulation of Fe acquisition from lactoferrin was slightly less: 4- to 7-fold (Table I). Two different methods of separating cell-associated from free Fe chelates (see Materials and Methods) produced similar results.

Ga induction of Fe acquisition from either transferrin or lactoferrin was concentration dependent (Fig. 2), a result consistent with our previous observations of Fe acquisition from NTA (50). Also similar to previous results with Fe-NTA (44), the divalent metal cations Ca2+, Cd2+, Mg2+, and Mn2+ did not affect acquisition of Fe from either transferrin or lactoferrin (Table I). However, in contrast to these earlier data (44), incubation of HL-60 cells with Cu2+ markedly increased Fe acquisition from both transferrin and lactoferrin (Table I). Zn2+ also exhibited a modest stimulatory effect (Table I).

The metals did not have to be continuously present in the extracellular medium during cellular exposure to transferrin or lactoferrin to induce Fe acquisition. Cells that were extensively washed to remove extracellular and loosely associated Ga before measurement of Fe acquisition exhibited a similar rate and magnitude of Fe acquisition from transferrin and lactoferrin as was observed if Ga remained throughout the course of Fe acquisition (Fig. 3). Thus, the metals did not increase Fe acquisition by displacing Fe from transferrin or lactoferrin in solution.

We then asked whether association of Ga with the plasma membrane is required to maintain the “induced phenotype” for acquisition of Fe from transferrin and lactoferrin. We incubated cells with varying concentrations of Ga(NO3)3 for 30 min at 37°C, then 750 nM [55Fe2+]transferrin or [55Fe2+]lactoferrin was added. Results are expressed as pmol of 55Fe acquired/106 cells after 60 min of incubation at 37°C (mean ± SEM, n = 3) with [55Fe2+]transferrin (○) or [55Fe2+]lactoferrin (□), as a function of increasing concentrations of Ga.

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**Table I. Enhancement of Fe transferrin by various metals**

<table>
<thead>
<tr>
<th>Cations</th>
<th>From transferrin</th>
<th>From lactoferrin</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.85 ± 0.35</td>
<td>7.42 ± 0.47</td>
<td>8</td>
</tr>
<tr>
<td>Ca</td>
<td>3.27 ± 0.62</td>
<td>8.30 ± 0.84</td>
<td>3</td>
</tr>
<tr>
<td>Cd</td>
<td>4.73 ± 0.46</td>
<td>9.74 ± 0.82c</td>
<td>3</td>
</tr>
<tr>
<td>Cu</td>
<td>47.86 ± 1.45c</td>
<td>52.83 ± 4.29c</td>
<td>3</td>
</tr>
<tr>
<td>Mg</td>
<td>3.71 ± 0.6d</td>
<td>12.47 ± 1.51d</td>
<td>3</td>
</tr>
<tr>
<td>Mn</td>
<td>3.50 ± 0.42</td>
<td>8.88 ± 0.85</td>
<td>3</td>
</tr>
<tr>
<td>Zn</td>
<td>7.33 ± 1.1d</td>
<td>12.69 ± 1.18d</td>
<td>3</td>
</tr>
<tr>
<td>Al</td>
<td>13.12 ± 0.13c</td>
<td>28.98 ± 0.70c</td>
<td>2</td>
</tr>
<tr>
<td>Fe</td>
<td>37.83 ± 0.14c</td>
<td>36.41 ± 0.99c</td>
<td>2</td>
</tr>
<tr>
<td>Ga</td>
<td>28.90 ± 2.07c</td>
<td>40.15 ± 1.18c</td>
<td>8</td>
</tr>
<tr>
<td>Cd</td>
<td>17.15 ± 0.10c</td>
<td>32.84 ± 0.07c</td>
<td>2</td>
</tr>
<tr>
<td>La</td>
<td>17.14 ± 0.68c</td>
<td>33.31 ± 0.20c</td>
<td>2</td>
</tr>
<tr>
<td>Sn</td>
<td>51.82 ± 0.27c</td>
<td>50.03 ± 3.30c</td>
<td>3</td>
</tr>
<tr>
<td>Zr</td>
<td>44.20 ± 2.64c</td>
<td>52.37 ± 2.06c</td>
<td>3</td>
</tr>
</tbody>
</table>

* a HL-60 cells (5 × 10⁶/ml) were incubated with various metal cations (1 mM) in HBSS and incubated for 30 min at 37°C after which [55Fe2+]transferrin or [55Fe2+]lactoferrin was added. Cell-associated [55Fe2+] was determined after incubation for 60 min at 37°C. Shown is the mean ± SEM of cell-associated [55Fe2+] (picomoles/10⁶ cells) acquired from transferrin or lactoferrin by control (non-metal-treated) cells and metal-treated cells. Statistically significant increases in cell-associated [55Fe2+] are designated by the footnotes below. All other data were not statistically significant, p > 0.05.

* b p < 0.01.
* c p < 0.0001.
* d p < 0.001.

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**FIGURE 1.** HL-60 iron acquisition from transferrin and lactoferrin is increased by gallium. A, HL-60 cells were suspended in HBSS following which 750 nM [55Fe2+]transferrin (○) or [55Fe2+]lactoferrin (□) were added. The cell suspension was then incubated for defined time periods at 37°C, after which cell-associated 55Fe was determined. B, Data were obtained under the same conditions except that the HL-60 cells were first incubated with 1 mM Ga(NO3)3 for 30 min before the addition of [55Fe2+]transferrin (○) or [55Fe2+]lactoferrin (□). The Ga remained throughout the experiment. In both panels cell-associated 55Fe is expressed as pmol of 55Fe acquired/10⁶ cells and the results shown are the mean ± SEM from 9–15 separate experiments.

**FIGURE 2.** Ga induction of increased Fe uptake is concentration dependent. HL-60 cells were incubated with varying concentrations of Ga(NO3)3 for 30 min at 37°C, then 750 nM [55Fe2+]transferrin or [55Fe2+]lactoferrin was added. Results are expressed as pmol of 55Fe acquired/10⁶ cells after 60 min of incubation at 37°C (mean ± SEM, n = 3) with [55Fe2+]transferrin (○) or [55Fe2+]lactoferrin (□), as a function of increasing concentrations of Ga.
Transferrin or lactoferrin was added to each group of cells and were prepared which were incubated initially in HBSS that did not contain Ga. Ga (50). When Fe acquisition from transferrin or lactoferrin was then measured, cell-associated Fe of the NTA-treated cells was not increased relative to non-Ga-treated control cells. After 120 min Fe acquisition from transferrin was 22.5 ± 4.4 and 19.8 ± 5.4 pmol/10^6 cells for control and Ga-treated cells, respectively, and 49.7 ± 7.3 and 60.5 ± 5.4 pmol/10^6 cells from lactoferrin by control and Ga-treated cells, respectively.

Because Fe initially chelated to low-m.w. compounds like NTA stimulates its own acquisition by HL-60 cells (44, 50), we examined the possibility that the difference in the basal rate of Fe acquisition from transferrin compared with lactoferrin (Fig. 1) related to differences in the ability of diferric transferrin and diferric lactoferrin to induce the cellular Fe acquisition mechanism. Surprisingly, HL-60 cells incubated with 100 μM Fe-lactoferrin for 30 min exhibited a decreased ability to acquire Fe from NTA relative to control cells (Fig. 4). In contrast, an enhancement in Fe acquisition from NTA was observed in cells pretreated with a similar concentration of Fe-transferrin (Fig. 4).

**Figure 3.** Ga-induced Fe uptake does not require the continued presence of Ga in solution. HL-60 cells were treated with 1 mM Ga(NO₃)₃ for 30 min at 37°C. The cells were divided into two groups. One was washed in HBSS to remove extracellular and loosely cell-associated Ga. A third group of control cells (untreated) were prepared which were incubated initially in HBSS that did not contain Ga. [⁵⁹Fe²⁺]Transferrin or [⁵⁹Fe²⁺]lactoferrin was added to each group of cells and incubated at 37°C for 120 min, following which cell-associated ^⁵⁹Fe was determined. Results are expressed as pmol (mean ± SEM, n = 3) of ^⁵⁹Fe/10^6 cells following 120 min of incubation with either [⁵⁹Fe²⁺]transferrin or [⁵⁹Fe²⁺]lactoferrin by untreated cells (dotted bars), cells that were washed after 30 min incubation with Ga (filled hatched bars), or cells in which Ga remained (open hatched bars) throughout the time of ^⁵⁹Fe incubation.

**Figure 4.** The amounts of Fe acquired from transferrin/lactoferrin are not related to ability of the chelates to induce an increase in Fe uptake. Cells were preincubated with 100 μM [Fe₃]transferrin (○), [Fe₃]lactoferrin (□), or buffer alone (□) for 30 min. The cells were washed and then incubated with 750 nM [⁵⁹Fe³⁺]NTA at 37°C. The cells were withdrawn at specified time and cell-associated ^⁵⁹Fe was assessed. Results are mean ± SD of two separate experiments.
values were 50.4 ± 6.3 and 55.6 ± 3.4 pmol Fe/10⁶ cells (mean ± SEM, n = 3) with control and cytoskeleton-treated cells, respectively. Thus, disruption of the cytoskeleton has no effect on Ga-stimulated Fe acquisition from transferrin.

**Gallium increases binding of transferrin and lactoferrin to HL-60 cells**

The classic process of cell acquisition of Fe from transferrin involves the binding of the Fe-transferrin complex to specific receptors on the cell surface. We asked whether the metal-induced increase in Fe acquisition from transferrin and lactoferrin also was associated with the enhanced binding of these Fe-protein complexes to the cell surface.

To test this hypothesis, control or metal-pretreated cells were incubated at 4°C with [¹²⁵I]diferric transferrin or [¹²⁵I]diferric lactoferrin for 15 min. Under these conditions, cell-associated [¹²⁵I]transferrin or [¹²⁵I]lactoferrin following Ga, Fe, Gd, Al, Sn, or Zr treatment cells was increased 10- to 42-fold over control cells (Table II). The tri- and tetravalent cations increased Fe acquisition and protein binding in roughly similar proportions. However, Cu exposure enhanced Fe acquisition from transferrin 17-fold (Table I) but had a much smaller effect on binding of transferrin protein (1.8-fold increase; Table II). The Cu effect on lactoferrin protein and Fe acquisition from lactoferrin was similar (7- to 8-fold increase; Tables I and II). Zn, which modestly enhanced Fe acquisition (Table I), also increased binding of both proteins only slightly (~4-fold; Table II). No increase in binding of either transferrin or lactoferrin was observed with Cd or Mn (Table II). The magnitude of binding was similar, regardless of whether studies were performed with apo- or iron-loaded transferrin or lactoferrin (Fig. 5). In contrast to the results with transferrin and lactoferrin, Ga exposure had a much lesser ability to enhance binding of [¹²⁵I]-labeled BSA to the cell surface (Fig. 5).

We next asked whether the Ga-induced increase in transferrin and lactoferrin protein association with HL-60 cells was dependent on continued Ga presence on the cell surface. As was the case with Fe acquisition from lactoferrin and transferrin, Ga-mediated enhancement of transferrin/lactoferrin protein binding was reversed when Ga was removed from the cells by chelation with NTA (50). Pretreatment of Ga-incubated cells with NTA reduced both transferrin (apo-transferrin and diferric transferrin) and lactoferrin protein binding back to near basal levels (Fig. 6). These results suggest that continuous association of Ga with the plasma membrane is required for enhanced transferrin and lactoferrin protein binding. They also suggest that the ability of Ga to increase Fe acquisition is mediated in part through its enhancement of transferrin and lactoferrin binding to the cell surface.

**Gallium does not increase transferrin receptor density on the plasma membrane**

HL-60 cells express transferrin receptors on their plasma membrane. Cycloheximide (1 mM for 60 min) pretreatment had no effect on Ga-mediated enhancement of transferrin or lactoferrin binding to HL-60 cells (data not shown), indicating Ga does not mediate its effects by increasing synthesis of transferrin receptor or any other protein. This is not surprising given that the time of incubation (30 min) required for Ga to produce its augmentation of transferrin and lactoferrin binding should not be sufficient to allow significant increases in protein synthesis.

Another possible explanation for increased transferrin and lactoferrin binding would be if Ga increased the number of transferrin receptors on the cell surface. Cells were exposed to Ga as above and were washed free of non-membrane-associated Ga. Ga-treated and control cells were then incubated with either of two different monoclonal anti-transferrin receptor Abs (A27.15 or E2.3), then stained with FITC-labeled secondary Ab, and surface expression of transferrin receptor was assessed by FACS analysis. There was no statistically significant difference in the level of transferrin receptor 1 on Ga-treated and control cells using monoclonal anti-transferrin receptor Ab (data not shown), indicating that Ga did not increase the number of surface transferrin receptors on HL-60 cells.

**Cellular retention of iron, transferrin, and lactoferrin**

We next sought to investigate whether the Fe that became cell associated reflected Fe that was taken up in association with the protein or that had been separated from it. Consequently, we compared the amount of protein relative to Fe that became cell associated as a consequence of incubating the cells with diferric transferrin or diferric lactoferrin.

Under basal conditions (Fig. 7A), the amount of Fe that became associated with the cells over 1 h at 37°C was ~2-fold greater when...
it was initially bound to lactoferrin relative to transferrin (Figs. 1A and 7A). Cell-associated lactoferrin protein was ∼3-fold higher than transferrin (Fig. 7A). Ga treatment led to a 10-fold increase in the amount of Fe acquired from transferrin and a 5-fold increase from lactoferrin after 1 h (Fig. 7, A vs B). This was mirrored by a similar magnitude of increase in cell-associated transferrin and lactoferrin protein as a consequence of Ga exposure (Fig. 7, A vs B) after 1 h of incubation.

There was about twice as much cell-associated Fe compared with transferrin protein with both non-Ga-treated and Ga-treated cells following incubation for 60 min at 37°C (Fig. 8). A similar ratio was seen with lactoferrin with non-Ga-treated cells. This approximates the expected molar ratio of fully Fe-loaded lactoferrin or transferrin of two Fe molecules to one protein molecule. With Ga-treated cells this 2:1 ratio was maintained for Fe-loaded transferrin, but for Fe-loaded lactoferrin the ratio fell to ∼1:1 (Fig. 8). A 2:1 ratio of Fe-transferrin protein association with the cells was also obtained when the incubations were performed at 4°C (Fig. 8). Although this was also true with lactoferrin for Ga-treated cells, the ratio of Fe: lactoferrin protein in the absence of Ga treatment was ∼5:1 (Fig. 8).

These data are consistent with a process that initially involves binding and retention of Fe as a complex with lactoferrin or transferrin. To examine whether all of the Fe initially associated with the cell remains complexed to lactoferrin or transferrin over the first 2 h of cellular acquisition, HL-60 cells (control or Ga-induced) were incubated in parallel with [59Fe]transferrin, [59Fe]lactoferrin, [125I]transferrin, or [125I]lactoferrin. The cells were washed and lysed, and then cell-associated 59Fe and 125I were obtained. Results using transferrin or lactoferrin and HL-60 cells preincubated with Ga(NO3)3 were measured. Results were expressed as pmol 59Fe vs 125I.

Relative uptake of [59Fe] and [125I]Tf/Lf by HL-60 cells at 37°C. HL-60 cells that had been treated with HBSS (control) or with 1 mM Ga were incubated with 750 nM [59Fe]transferrin, [59Fe]lactoferrin, [125I]diferric transferrin, or [125I]diferric lactoferrin at 37°C for 2 h. The cells were washed and cell-associated 59Fe and 125I were obtained. Results are expressed in pmol (mean ± SEM, n = 3) 59Fe (open hatched bars) or pmol (mean ± SEM) 125I (filled hatched bars) per 10^6 cells (n = 3). A. Results after 120 min of incubation with transferrin (Tf) or lactoferrin (Lf) using HL-60 cells not exposed to Ga. B. Results using transferrin or lactoferrin and HL-60 cells preincubated with Ga(NO3)3.

Retention of Fe by HL-60 cells. HL-60 cells (4 × 10^6/ml) were incubated with 1 mM Ga(NO3)3 or HBSS (control) for 30 min followed by incubation with 1 μM [59Fe]transferrin, [59Fe]lactoferrin, or [59Fe]NTA for 2 h at 37°C. The cells were centrifuged and the cell pellets were washed three times in HBSS at 4°C. Each group of cells was divided into two groups of triplicate samples. One group was assayed for cell-associated 59Fe by a gamma counter. The other group was resuspended in culture medium at 2 × 10^6/ml and placed in the incubator at 37°C for 18 h. Cells were centrifuged at 500 × g for 10 min at 4°C. Medium was carefully removed and the amounts of cell-associated and medium-containing 59Fe were measured. Results were expressed as pmol (mean ± SEM, n = 3–5) 59Fe per 10^6 cells for untreated (filled bars) and Ga-induced cells after 2 h (dotted bars) and then untreated (striped bars) and Ga-induced cells 18 h later (open hatched bars) as a function of the Fe chelate (transferrin, lactoferrin, or NTA) from which they acquired the Fe.

FIGURE 7. Relative uptake of [59Fe] and [125I]Tf/Lf by HL-60 cells at 37°C. HL-60 cells that had been treated with HBSS (control) or with 1 mM Ga were incubated with 750 nM [59Fe]transferrin, [59Fe]lactoferrin, [125I]diferric transferrin, or [125I]diferric lactoferrin at 37°C for 2 h. The cells were centrifuged at 500 × g for 10 min at 4°C. Each group of cells was divided into two groups of triplicate samples. One group was assayed for cell-associated 59Fe and 125I were obtained. Results are expressed in pmol (mean ± SEM, n = 3) 59Fe (open hatched bars) or pmol (mean ± SEM) 125I (filled hatched bars) per 10^6 cells (n = 3). A. Results after 120 min of incubation with transferrin (Tf) or lactoferrin (Lf) using HL-60 cells not exposed to Ga. B. Results using transferrin or lactoferrin and HL-60 cells preincubated with Ga(NO3)3.

FIGURE 8. Relative uptake of [59Fe] and [125I]Tf/Lf by HL-60 cells at 4°C. HL-60 cells that had been treated with HBSS (control) or with 1 mM Ga were incubated with 750 nM [59Fe]transferrin, [59Fe]lactoferrin, [125I]diferric transferrin, or [125I]diferric lactoferrin at 4°C or 37°C for 1 and 2 h, respectively. The cells were washed and cell-associated 59Fe and 125I were obtained. Results are expressed as the ratio of cell-associated pmol per 10^6 cells 59Fe to 125I (n = 3). Open hatched bars represent results of cells under basal conditions, and filled hatched bars represent cells that had been preincubated with Ga(NO3)3.

FIGURE 9. Retention of Fe by HL-60 cells. HL-60 cells (4 × 10^6/ml) were incubated with 1 mM Ga(NO3)3 or HBSS (control) for 30 min followed by incubation with 1 μM [59Fe]transferrin, [59Fe]lactoferrin, or [59Fe]NTA for 2 h at 37°C. The cells were centrifuged and the cell pellets were washed three times in HBSS at 4°C. Each group of cells was divided into two groups of triplicate samples. One group was assayed for cell-associated 59Fe by a gamma counter. The other group was resuspended in culture medium at 2 × 10^6/ml and placed in the incubator at 37°C for 18 h. Cells were centrifuged at 500 × g for 10 min at 4°C. Medium was carefully removed and the amounts of cell-associated and medium-containing 59Fe were measured. Results were expressed as pmol (mean ± SEM, n = 3–5) 59Fe per 10^6 cells for untreated (filled bars) and Ga-induced cells after 2 h (dotted bars) and then untreated (striped bars) and Ga-induced cells 18 h later (open hatched bars) as a function of the Fe chelate (transferrin, lactoferrin, or NTA) from which they acquired the Fe.
indicating that these data were not the result of dislodging $^{59}$Fe from the protein during the immunoprecipitation procedure. Both Fe and protein that became cell associated appeared to remain cell associated over a prolonged time period. Control and Ga-treated HL-60 cells were incubated with $^{59}$Fe-lactoferrin or $^{59}$Fe-transferrin for 2 h, following which the cells were washed and placed back into culture in the absence of $^{59}$Fe, lactoferrin, or transferrin. At 18 h the amount of $^{59}$Fe retained by the cells was ascertained. As shown in Fig. 9, $\sim 60\%$ of Fe initially acquired from transferrin and lactoferrin remained associated with the cell 18 h later. This was true for both control and Ga-induced cells (Fig. 9). Similar results were obtained when Fe acquisition from the low-m.w. chelating agent NTA was assessed in a similar manner (Fig. 9).

Discussion

We have previously provided evidence for the existence of a multivalent metal-inducible high-capacity mechanism for Fe acquisition from low-m.w. chelates by human myeloid cells that functions even under conditions of extremely low ATP availability (44, 50). In the present work, using HL-60 cells as a model system, we now find that a mechanism with many similar features allows for the acquisition of Fe from both transferrin and lactoferrin. Under basal conditions, cellular Fe acquisition from both lactoferrin and transferrin was demonstrable, with Fe acquisition from lactoferrin somewhat greater than that from transferrin. Exposure of HL-60 cells to a variety of trivalent and tetravalent metals, including Fe, dramatically increased the magnitude of Fe acquisition from both Fe-binding proteins. The majority of the initially cell-associated Fe was still present 18 h later, irrespective of whether the Fe was acquired from transferrin or lactoferrin. Thus, the Fe acquired by this mechanism does not reflect a transient association with the cell surface. Interestingly, nearly all of the increase in Fe acquisition occurred in the first 10 min, which then reached a plateau. This is identical to what we have previously observed with Fe acquisition by myeloid cells from a variety of low-m.w. Fe chelating agents following multivalent metal exposure (44). The reason for these kinetics is unclear. It appears that the interactive sites were almost completely saturated within 30 min in metal-induced cells. The molecular nature of these sites remains to be defined. However, the process does not require endocytosis because it was unaffected by depleting the cells of ATP or disrupting the cytoskeleton.

The ability of Fe and other metals to enhance Fe acquisition from transferrin has been previously reported for some nonmyeloid cell types (17–20, 57). For example, ferric citrate modestly enhanced Fe acquisition from transferrin by hepatocytes (17), SK-MEL-28 melanoma cells (18, 19, 57), Chinese hamster ovary cells (18), and lens epithelial cells (20). Where examined this process could not be attributed to enhanced transferrin binding to the cell. Gallium enhanced Fe acquisition from transferrin by SK-MEL-28 melanoma cells but was less effective at doing so than Fe (57). To our knowledge the impact of other metals on cellular Fe acquisition from transferrin has not been examined. Minimal data are available regarding the impact of Fe or Ga on Fe acquisition from lactoferrin. Fe reportedly increases uptake of Fe bound to lactoferrin by hepatocytes (58). In contrast, depletion of intracellular Fe increased acquisition of Fe from lactoferrin by a human colon carcinoma cell line (36). In contrast to results with the trivalent and tetravalent metals, we found that most divalent metals lacked the ability to modulate Fe acquisition from either transferrin or lactoferrin. However, Cu and to a lesser extent Zn increased cellular Fe acquisition from both transferrin and lactoferrin. This was somewhat surprising, as we had previously found that Cu and Zn had no effect when acquisition of Fe bound to low-m.w. chelates was studied (44). This anomalous behavior of Cu and Zn remains unexplained and stands in contrast to an absence of an effect of Cu and Zn on Fe acquisition from transferrin by SK-MEL-28 melanoma cells (57).

How these various metals enhance cellular Fe acquisition also remains unclear. Ga and other metals can bind to the Fe-binding sites of transferrin and lactoferrin (59–62). Therefore, one explanation could have been displacement of Fe from the binding site(s) on transferrin/lactoferrin by these metals. This would increase the concentration of free extracellular Fe available for acquisition via a transferrin-independent mechanism. If this displacement of Fe occurred in solution, the increased concentration of free Fe might have contributed to the increased Fe acquisition. However, this scenario seems very unlikely because there was no difference in Fe acquisition when extracellular Ga was removed before the measurement of Fe uptake (Fig. 3). The effect of Ga (and presumably other metals) must be related to its interaction with the cell.

Fe reduction has been postulated to play a key role in the acquisition of transferrin- and non-transferrin-bound Fe by yeast and some other eukaryotic cells. In addition, Cu regulates Fe uptake from low-m.w. complexes in both yeast and some human cells through its role in enzymes with ferroxidase activity that contribute to Fe acquisition (45, 63–65). However, we have previously shown that Fe reductase activity in HL-60 cells is not altered by exposure to Ga and other metals and, thus, the ability of the trivalent and tetravalent metals to alter cellular Fe acquisition cannot be due to effects on Fe reductase activity (50). Others have in fact reported that monocyte-macrophage ferric reductase activity decreases following exposure to exogenous Fe (66).

Copper, like Fe, is redox active and can serve as a catalyst for the formation of toxic oxidant species such as the hydroxyl radical (67). It has been suggested that formation of such oxidant species may be involved in the ability of these metals to increase Fe acquisition in some cells (18, 20). However, this seems an unlikely explanation for the trivalent metal effects overall, because a number of the metals observed to increase Fe acquisition, e.g., Ga and Gd, are unable to redox cycle or catalyze oxidant production under biologic conditions. We find no evidence for hydroxyl radical formation using spin trapping techniques as a consequence of cellular exposure to 1 mM Ga(NO$_3$)$_3$ (O. Olakanni, J. B. Stokes, G. T. Rasmussen, and B. E. Britigan, unpublished observation).

Because both transferrin and lactoferrin become associated with the cellular membrane of myeloid cells (28, 32–37, 51, 52), we hypothesized that the mechanism through which multivalent metals enhance Fe acquisition from transferrin and lactoferrin could be the result of an increase in binding of these proteins to the cell surface. Studies using $^{125}$I-labeled transferrin and lactoferrin confirmed that exposure of HL-60 cells to each of the trivalent and tetravalent metals that increased cellular Fe acquisition from lactoferrin and transferrin also dramatically increased binding of these two proteins to the cell surface (Table II). In contrast, the divalent metals that did not increase cellular Fe acquisition also did not enhance protein binding. However, Cu, which increases cell-associated Fe to an extent similar to that seen with the trivalent and tetravalent metals, results in a much smaller increase in lactoferrin or transferrin binding. Thus, with the exception of the effect of Cu, the ability of different metals to enhance Fe acquisition from transferrin and lactoferrin correlates with their ability to enhance the association of these proteins with the cell surface.

For most cell types, extracellular Fe exposure leads to a decrease in transferrin binding through a decrease in surface expression of transferrin receptor (9–11). Macrophages appear to be an exception to this rule (51). In contrast to our results, Richardson et al. (17) did not find any increase in transferrin binding to hepatocytes as a result of ferric citrate exposure, a process that nevertheless
enhanced cellular Fe acquisition from transferrin up to 8-fold. Fe exposure has previously been reported to enhance binding of lactoferrin to rat hepatocytes (58) and the immortalized human respiratory epithelial BEAS-2B cell line (68). In contrast, depletion of intracellular Fe also enhanced lactoferrin binding to the colon carcinoma HT29–18-C1 cell line (36). Whether our data that Ga and other metals enhance cellular binding of transferrin and lactoferrin extend to other cell types requires further study.

Specific receptors for transferrin and lactoferrin have been reported to be present on the surface of myeloid cells (9–11, 28, 30, 32–39). The transferrin receptor has been well described (9–11); however, the identity of cellular lactoferrin receptor(s) remains poorly defined (28, 30, 32–39). Our data do not suggest that the mechanism responsible for the ability of the various metals to enhance surface binding of these proteins to the cell surface involves an increase in transferrin receptor expression. FACS analysis showed no change in transferrin receptor expression as a function of trivalent metal exposure. Previous work (69) has shown that exposure of HL-60 cells to Ga in the form of Ga-transferrin does lead to an increase in transferrin receptor surface expression. However, this takes several days compared with the brief (<1 h) Ga(NO₃)₃ exposure used in our studies. Furthermore, transferrin receptor expression in HL-60 cells has been reported to be in the range of 26,000–114,000 sites per cell, depending upon the extent to which the cells have reached confluence growth (69). Thus, the number of transferrin receptors reported to be expressed on the surface of HL-60 cells would be nearly two orders of magnitude less than amount of transferrin binding per cell that we detected after Ga treatment. Because multiple cellular receptors of varying specificity have been reported to exist for lactoferrin (28, 30, 32–38, 70–72) it is difficult to definitively approach the cell “receptor” that leads to enhanced lactoferrin binding following cellular exposure to multivalent metals. To our knowledge, extensive studies of the “receptor(s)” whereby lactoferrin binds to HL-60 cells have not been performed.

Despite the similarities between Fe acquisition from transferrin and lactoferrin, there are several differences. The magnitude of binding of lactoferrin protein was severalfold greater than transferrin binding properties and their interaction with cellular membranes. Such differences include their isoelectric points (~9 for lactoferrin and 5–5.5 for transferrin) (73) and differences in glycosylation (10, 74).

Regardless of whether the cells were studied in the basal state or after Ga exposure, the amount of Fe that initially became cell associated was close to the 2:1 molar binding ratio of Fe:transferrin and Fe:lactoferrin. These data are consistent with a process that initially involves binding and retention of Fe as a complex with lactoferrin or transferrin. DMT-1 is expressed in most cell types (75), including macrophages (76–79), making it a candidate for involvement in the eventual removal of Fe from transferrin or lactoferrin in the system we have described. DMT-1 is expressed on the surface of intestinal epithelial cells (80). Although DMT-1 has been detected in macrophage early endosomes (79), it is not known whether it is similarly localized on the plasma membrane of myeloid cells. However, the events that lead to the subsequent internalization, storage, or secretion of Fe and/or transferrin and lactoferrin by these cells over time require further delineation.

In summary, HL-60 cells possess a high-capacity multivalent metal-inducible mechanism for Fe acquisition from transferrin and lactoferrin that bears many similarities to the process previously described (44), which allows these and other cells types to acquire Fe from low-m.w. Fe chelates. This acquisition is not via the classic transferrin receptor pathway. The potential biologic importance of this mechanism may relate to its high Fe acquisition capacity, with which it is able to rapidly respond to an increased level of extracellular Fe. In a condition of acute Fe overload in which rapid Fe sequestration may be desirable, cells that function in Fe storage, such as macrophages, would benefit from a mechanism with rapid activation and high capacity. Evidence presented here suggests a role for increased surface binding of transferrin and lactoferrin to the cell surface in the ability of metals to enhance Fe acquisition from these proteins. Further work is required to delineate the mechanism and cellular consequences of Fe acquired from these two key Fe-binding proteins via this process as well as the extent to which it is present in other cell types.

References