Polyvalent Cationic Metals Induce the Rate of Transferrin-independent Iron Acquisition by HL-60 Cells*

Oyebode Olakanmi, John B. Stokes, Shadab Pathan, and Bradley E. Britigan‡

From the Research Service, VA Medical Center, Iowa City, Iowa 52246 and Department of Internal Medicine, The University of Iowa College of Medicine, Iowa City, Iowa 52242

The trivalent metals iron, aluminum, and gallium greatly increase the rate of iron acquisition from low molecular weight chelates by human myeloid cells. The present study explores the mechanism responsible. Gallium-induced iron acquisition was shown to lead to stable cellular association of iron, the magnitude of which varied with the chelate to which the iron was bound. The majority of this iron initially associated with the plasma membrane. Cellular depletion of ATP did not affect the response to gallium nor did it require the continued presence of extracellular gallium. However, continued cell association of gallium was needed as subsequent cellular exposure to metal chelators resulted in a rapid loss of the “induced” phenotype. Other trivalent metals (lanthanum and gadolinium) and tetravalent metals (tin and zirconium) but not divalent metals also induced iron acquisition. Neither enhanced iron reduction nor protein kinase C or tyrosine kinases appeared involved in gallium-mediated induction of iron acquisition. Exposure of HL-60 cells to polyvalent cationic metals results in a dramatic and sustained increase in the rate of iron acquisition from low molecular weight chelating agents. This could be important for the rapid clearance of iron by phagocytes from the extracellular environment at sites of local tissue damage.

Iron is essential for most metabolic processes in living cells but is also potentially cytotoxic due to its ability to promote the formation of toxic oxidants such as the hydroxyl radical (1). Consequently, nearly all cellular systems have evolved mechanisms that allow them to tightly regulate their intracellular concentrations of this metal (1, 2). In addition, some cell types may have evolved means to regulate extracellular iron levels so as to limit the potential cytotoxicity of iron for adjacent cells (3, 4).

Macrophages play an important role in regulating the availability of iron for other mammalian cells by serving as an iron storage site (5). Acquisition of iron bound to transferrin via receptor-mediated endocytosis has been the mechanism of iron acquisition most extensively studied in macrophages and other cell types (2, 6–10). However, macrophages and other myeloid cells possess a high capacity transferrin-independent mechanism for iron acquisition from a variety of low molecular weight iron chelates (11–14). The exact mechanism is not yet known, but does not involve endocytosis or pinocytosis (14). Furthermore, this system can acquire iron at a rate far in excess of transferrin receptor-mediated endocytosis (14).

One important additional feature of the macrophage transferrin-independent iron acquisition mechanism is that iron markedly stimulates the rate of its own acquisition (14). Consistent with a previous report with the HL-60 myeloid cell line (12), we found that the trivalent metals gallium and aluminum also exhibited the capacity to markedly induce the rate of iron acquisition from nitrilotriacetate (NTA)¹ by macrophages and other myeloid cells (14). However, the mechanism of induction of increased iron uptake resulting from exposure to these trivalent metals remained undefined.

Under conditions of iron overload, transferrin becomes saturated, and its capacity to remove extracellular iron is overwhelmed (15, 16). Under such conditions, the concentration of small molecular weight iron chelates may increase. Thus, increased iron uptake by the transferrin-independent mechanism of macrophages may be an important means of rapid clearance of cytotoxic iron and protection against potential iron-mediated tissue injury. Given the potential importance of this second mechanism of iron acquisition by myeloid cells, we performed a series of experiments designed to further characterize and define the mechanism and metal-mediated regulation of transferrin-independent iron acquisition by myeloid cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The human promyelocytic HL-60 cell line was cultured in RPMI (University of Iowa Cancer Center, Iowa City, IA) containing 10% serum plus glutamine (2 mM, Sigma), penicillin, and streptomycin (14).

**Preparation of Iron Chelates**—Unless otherwise stated, [59Fe]Cl₃ (Amersham Corp., 100 μCi/ml) was added in a 1:1 molar ratio to the desired low molecular weight chelator. [67Ga]Citrate was obtained from Mallinkrodt Chemical Works and diluted to the desired concentration prior to use. Chelates employed included NTA (Sigma), EDTA (Mallinkrodt), citrate (Fisher), ascorbate (Sigma), ADP (Sigma), glycyl-L-histidyl-L-lysine (GHL, Aldrich), diethylentriaminepentaaetate (Sigma), and deferoxamine (Sigma).

**Quantitation of Cellular Iron or Gallium Acquisition**—Cells were washed three times in MEM-α (University of Iowa Cancer Center, Iowa City, IA), resuspended in the same medium at 5 × 10⁶/ml, and then placed in a 96-well plate at 100 μl/well for 15 min (5% CO₂, 37 °C). The desired ⁵⁹Fe or ⁶⁷Ga chelate (740 μM) was then added. After the desired period of ⁵⁹Fe or ⁶⁷Ga exposure, the cell suspension was centrifuged at 500 × g for 5 min (4 °C) and the medium was carefully aspirated. At this point, the cell pellet was dissolved in a solution containing 40 μl of 1 N NaOH and 10 μl of 10% trichloroacetic acid. The iron or gallium in the supernatant was quantitated by atomic absorption spectrophotometry. The iron or gallium remaining in intact cells was determined by extracting with 1 N NaOH and 10% trichloroacetic acid and measuring the amount of radioactivity in the supernatant. The results are expressed as percentage of ⁵⁹Fe or ⁶⁷Ga chelate incorporated per cell. The statistical significance was evaluated by the t test.

¹ The abbreviations used are: NTA, nitrilotriacetate; GHL, glycyl-L-histidyl-L-lysine; MEM-α, minimal essential medium-α; BPS, bathophenanthrolane sulfonate; FZ, Ferrozine.
temperature, cellular iron acquisition is negligible (14). The cell pellet in each well was washed three times in the same volume of MEM-α and the amount of cell-associated $^{59}$Fe or $^{67}$Ga was determined by gamma counter. Parallel experiments were performed each day in the absence of cell to control for possible $^{59}$Fe binding to the plate or formation of non-cell associated iron or gallium aggregates which might have co-sedimented with the cells. These values, usually between 0.05 and 0.2% of total counts/min added, were subtracted from corresponding experimental samples for each time interval.

In some experiments cells were incubated with gallium or other metals for defined time periods (usually 30 min) at 37 °C prior to measurement of $^{59}$Fe or $^{67}$Ga acquisition. In other studies cells were incubated with various metabolic pathway inhibitors (NaCN (1 mM) and 2-deoxy-D-glucose (50 mM)) for 1 h at 37 °C. The medium was removed and the cells resuspended in fresh medium containing the inhibitors. After 15 min of incubation at the same temperature, iron or gallium acquisition was measured as above.

**Measurement of Cellular Cellular ATP—** Cells ($2 \times 10^6$/ml) were washed as described above and then incubated with a combination of NaCN (1 mM) and 2-deoxy-D-glucose (50 mM) for 1 h (37 °C). At the end of incubation period the cells were centrifuged ($500 \times g$, 10 min, 4 °C). Six percent cold perchloric acid was added to the cell pellets ($2 \times 10^6$/ml), which were then allowed to stand for 15 min at 4 °C. The suspension was centrifuged (12,000 $\times g$, 15 min, 4 °C). The supernatant was withdrawn into an Eppendorf tube and the acid was neutralized with 4 M KOH and 0.5 M imidazole. The solution was kept frozen at −80 °C until analyzed. The cell pellets were solubilized with 0.2% SDS in 1 N NaOH (pH 11.2) for protein determination. Cellular ATP was measured by the luciferin-luciferase method with use of a luminometer (Analytical Luminescence Laboratories, San Diego, CA) in accordance with the manufacturer’s instructions. ATP content was expressed as nanomoles of ATP per milligram of protein, with protein content being determined by the method of Avruch and Wallach (17).

**Measurement of Reductase Activity—** Assessment of cellular reduction of ferricyanide was carried out in Hanks’ balanced salt saline according to the method of Inman et al. (18). Cells ($4 \times 10^6$/ml) were incubated with 1 mM ferricyanide in the presence or absence of the desired trivalent metal, at 37 °C with agitation. At specific time intervals, 1 ml of the cell suspension was withdrawn and centrifuged. The optical density of the spent medium was obtained at 420 nm. In other experiments, reduction of ferric iron chelated to NTA was measured in the presence of 1 mM bathophenanthroline sulfate (BPS) or Ferrozine (FZ), and the formation of the [Fe$^{2+}$]BPS or [Fe$^{3+}$]FZ complex was measured spectrophotometrically (3, 19).

**Chemical and Enzymatic Modification—** Cells ($1 \times 10^7$/ml) were desialylated by incubation with neuraminidase (0.25 unit/ml, Boehringer Mannheim GmbH, Germany) at 37 °C for 1 h. The cells were washed three times and then exposed to the trivalent metal of interest before the measurement of iron acquisition. The activity of neuraminidase was assessed with thiobarbituric acid assay of released sialic acids (20). Similarly, sulfhydryl groups on the cell membrane were modified by incubation with 5 mM solution of either iodoacetic acid, N-ethylmaleimide, p-chloromercuribenzenesulfonic acid, or 5,5′-dithiobis(2-nitrobenzoic acid) at 37 °C for 15 min. The cells were washed twice prior to induction, and measurement of cellular iron acquisition was subsequently determined.

**Cell Fractionation—** Cells were incubated in 740 nM $[^{59}$Fe]NTA or $[^{59}$Fe]GHL in the presence or absence of 1 mM Ga(NO$_3$)$_3$, for 1 h at 37 °C. The cells were washed free of non-cell-associated $^{59}$Fe. The cells were then cavitated and fractionated into membrane and cytosolic components as described previously (3) following which $^{59}$Fe associated with each fraction was determined by gamma counter.

**RESULTS**

**Induction of Iron Acquisition by Metal Ions—** We (14) and other laboratories (12, 21, 22) have reported that, prior exposure to iron, gallium or aluminum increases the rate of the acquisition of iron from low molecular weight chelating agents by HL-60 and other myeloid cells (14). However, the mechanism responsible for the ability of these trivalent metals to mediate this induction process is not known. Although iron is the most physiologically relevant of these metals, it is technically difficult to assess if a compound induces its own rate of acquisition. Therefore, we investigated the mechanism of gallium-induced iron acquisition by HL-60 cells as a model for the iron-induced process in these and other myeloid cells.

First, we determined the optimal experimental conditions for gallium-induced iron acquisition from NTA by HL-60 cells. Dose response studies showed that exposure of HL-60 cells to a gallium concentration of 1 mM for 30 min leads to maximal induction of iron acquisition (Fig. 1A). Shorter incubation times led to lesser induction even at gallium concentrations in excess of 1 mM (Fig. 1B). Preincubation for longer time periods with lower gallium concentrations produced less than maximal induction (Fig. 1B).

**Iron Acquisition Varies with the Chelating Agent to Which the Iron Is Bound—** We next examined the effect of the chelate to which $^{59}$Fe was complexed on the ability of gallium to increase the rate of HL-60 acquisition of iron. As shown in Fig. 2, 1 mM gallium increased the rate of iron acquisition from each
chelating agent examined. However, the magnitude of the rate of iron acquisition varied with the iron chelate used, just as we had previously observed in the basal (uninduced) state (14). With each of the iron chelates examined a plateau of cell-associated iron was seen after 40–60 min (Fig. 2). This plateau was not the result of depletion of 59Fe from the system as the amount of 59Fe initially added was always considerably greater than that which became cell-associated.

Stability of Iron Association with HL-60 Cells after Acquisition—If gallium exposure truly increases cellular iron acquisition, the iron should remain cell-associated. To determine the extent to which acquired 59Fe could be dissociated from the gallium-induced HL-60 cells, wash out experiments were conducted. Cells were incubated with 59FeNTA in the presence or absence of 1 mM gallium for 30 min. Non-cell-associated 59Fe was removed by repetitive washing (three washes, 4 min/wash, 4°C), and the cells were resuspended in iron-free medium at 37°C. At various time intervals, aliquots were removed and both cell-associated and medium-containing 59Fe were determined. As shown in Fig. 3, cell-associated 59Fe remained stable over 2 h of incubation at 37°C after an initial release of approximately 20% in the first 10 min. Although much greater amounts of iron were associated initially with gallium-induced cells, both induced and noninduced cells retained similar percentages of initial cell-associated 59Fe. These studies thus indicate that iron acquired by HL-60 cells after gallium induction does not readily dissociate from the cell.

We next examined the cellular location of newly acquired iron. Control and gallium-induced HL-60 cells were incubated with 59FeNTA or 59FeGHL following which they were washed and subjected to subcellular fractionation. As shown in Table I, both the absolute amount and the proportion of 59Fe associated with the membrane-containing subcellular fractions was greater in gallium-induced cells than in the control cells. However, consistent with our previous data (14), a high percentage of newly acquired iron was associated with the membrane-containing compartment even in the control cells.

Depletion of ATP or Inhibition of Protein Synthesis Does Not

| Chelates  | 59Fe acquired | 11,000 × g pellet | 100,000 × g pellet | 100,000 × g supernatant |
|----------|---------------|------------------|--------------------|------------------------|
| [59Fe]NTA (uninduced) | 23.6 (1.17) | 8.3 (0.41) | 33.6 (1.67) | |
| [59Fe]NTA (Ga-induced) | 70.9 (26.9) | 11.0 (4.2) | 19.0 (2.7) | |
| [59Fe]GHL (uninduced) | 22.0 (6.6) | 8.7 (2.61) | 10.3 (3.09) | |
| [59Fe]GHL (Ga-induced) | 72.9 (37.3) | 12.0 (6.2) | 17.0 (8.9) | |

* Numbers in parentheses are the actual amount of 59Fe (pmol 59Fe) measured in each fraction per 10^6 cell equivalence.

Prevent Induction—Basal (uninduced) iron acquisition from NTA by myeloid cells is independent of the level of cellular ATP and active protein synthesis (14). However, it was not known whether the gallium-mediated induction process was also energy independent. When cells were treated with 1 mM NaCN and 50 mM 2-deoxy-D-glucose (dual inhibition of glycolysis and oxidative phosphorylation), there was a 98% decrease in cellular levels of ATP (Fig. 4). However, depletion of ATP had no effect on the ability of gallium to induce iron acquisition from NTA or citrate (Fig. 4). Inhibition of protein synthesis by cycloheximide (1 mM for 60 min.) also had no effect on the ability of gallium to increase the rate of iron acquisition from NTA (not shown).

Stability of Cellular Induction by Gallium—Having shown that the HL-60 cell iron acquisition rate dramatically increases in the presence of gallium, the extent to which extracellular gallium needed to be present for the maintenance of the induced state was assessed. HL-60 cells were incubated in 1 mM Ga(NO3)3 for 30 min following which extracellular gallium was removed. The cells were suspended in gallium-free medium,
and their ability to acquire \(^{59}\)Fe from NTA was measured at defined time points. Cells freshly removed from Ga(NO\(_3\))\(_3\) exhibited identical iron acquisition kinetics relative to those in which extracellular gallium was maintained (Fig. 5). Over the next 4 h, a slow decrease in the rate of iron acquisition was noted. However, even 4 h after gallium removal, acquisition of \(^{59}\)Fe from NTA remained about 60% of that of the cells which were continually exposed to Ga(NO\(_3\))\(_3\) (Fig. 5). Thus, once the iron acquisition system is induced, it is maintained in this state for several hours unless removed by chelators.

**Role of Valence of the Metal in Iron Induction**—In previous work, low concentrations of the trivalent metals gallium, aluminum, and iron, but none of a variety of divalent metals (zinc, cadmium, copper, and manganese) increased the rate of macrophage iron acquisition (14). Unlike iron, aluminum and gallium are not reducible under physiologic conditions. These data suggested that induction of myeloid cell iron acquisition may be a function of the valence of the metal to which the cells are exposed. Extending our earlier data (14), pre-exposure (30 min) of HL-60 cells with a 1 mM concentration of divalent metal salts (copper, cadmium, manganese, and zinc) was associated with only a modest (less than 2-fold) increase in iron acquisition from NTA or citrate (data not shown). In contrast, cells pre-exposed to a 1 mM concentration of the trivalent metals iron, aluminum, lanthanum, or gadolinium each induced iron acquisition from NTA by HL-60 cells to a similar or greater extent than that observed with gallium (Fig. 6). Following cell exposure to lanthanum and gadolinium, iron acquisition was 1.6—1.8 as great as with gallium. The concentration of each metal required for maximum induction was similar to that with gallium (1 mM). Tetravalent metals (tin or zirconium, 1 mM) induced iron acquisition at least as well if not better than lanthanum or gadolinium (Fig. 6). In order to assess whether induction of iron acquisition resulted from exposure to any multivalent cation, polylysine. This treatment had no effect on iron uptake whatsoever (Fig. 6). These data suggest that the ability to markedly increase the rate of iron acquisition by HL-60 cells is limited to trivalent and tetravalent metal salts.

**Ability of Chelated Metals to Induce the Rate of Iron Acquisition**—In both gallium-induced and control cells, iron acquisition by myeloid cells is dependent on the agent to which iron is chelated (14) (Fig. 2). Therefore, we examined the extent to which the chelating agent influenced a metal's capacity to induce the rate of HL-60 cell iron acquisition. Like the rate of iron acquisition from these same chelates, the potency of individual iron chelates to increase the rate of HL-60 cell acquisition of \(^{59}\)Fe from NTA is also chelate dependent (Fig. 7). Cells exposed to some ferric iron chelates (Fe-GHL, Fe-ADP) resulted in higher rates of iron acquisition from NTA than those treated with Ga(NO\(_3\))\(_3\). Other iron chelates (e.g., Fe-EDTA or Fe-deferoxamine) appeared to be very poor inducers of the iron acquisition mechanism.

In contrast to the iron results, if HL-60 cells were exposed to gallium, aluminum, gadolinium, or lanthanum which was first
chelated by NTA or citrate, no induction of iron acquisition was observed (not shown). In fact, additional studies with gallium indicated that many chelates prevented gallium induction. We next asked whether these chelating agents could reverse the induction resulting from previous exposure of the cells to Ga(NO₃)₃. HL-60 were exposed to gallium (NO₃)₃ (1 mM for 30 min) following which 0.5 mM NTA, citrate, ascorbate, or ADP were added for 10 min and then removed by washing. Analysis of the observed rate of subsequent acquisition of ⁵⁹Fe from NTA by these cells indicated the gallium-mediated induction of iron acquisition was rapidly reversed by exposure to each of the metal chelators employed. Iron acquisition was decreased to 25, 18, 48, and 60% of the gallium-treated control by NTA, citrate, ascorbate, and ADP, respectively. The extent of the reversal process was dependent on the concentration of the chelator employed with the threshold for the effect with NTA in the range of 100 μM.

It seemed most likely that the ability of these various metal chelating agents to reverse gallium-mediated induction of iron acquisition is that they remove gallium from a critical cellular site necessary to maintain the "induced" phenotype. Subsequent analysis supported this hypothesis. HL-60 cells were incubated with 740 nM [⁶⁷Ga]citrate for 60 min following which the cells were repetitively washed and then resuspended in the presence or absence of 100 μM NTA or citrate. Incubation of these cells with NTA or citrate resulted in a rapid (10 min) loss of nearly 50% of cell-associated ⁶⁷Ga with a slower decrease over 2 h (Fig. 8). In the absence of exposure to the chelating agent, cell-associated ⁶⁷Ga was unchanged over 2 h (Fig. 8). These data suggest that various metal-chelating agents are capable of removing gallium from a cellular compartment necessary to maintain the cell at its maximum capacity for iron acquisition.

Gallium Also Induces Its Own Acquisition—Previous work by Chitambar and Sax (12) suggested an interrelationship between gallium and iron acquisition by HL-60 cells. Consistent with their observations, we found that the kinetics of HL-60 acquisition of ⁶⁷Ga from citrate was similar to that observed for ⁵⁹Fe if the cells were not previously induced (Fig. 9). Furthermore, preincubation of HL-60 cells for 30 min with 1 mM Ga(NO₃)₃ greatly increased uptake of ⁶⁷Ga by these cells (Fig. 9). The kinetics of this induced state for gallium acquisition is also similar to that for iron acquisition after gallium induction (compare Figs. 2 and 9).

Importance of Redox State of Iron on Its Acquisition by HL-60 Cells—Several reports using other cellular systems, have suggested that acquisition of ferric iron (Fe³⁺) from non-transferrin chelates is coupled to its reduction to ferrous iron (Fe²⁺) (18, 23–25). This was based on the findings that iron uptake by these cells was inhibited by the presence of ferrous iron chelators such as BPS or FZ. Consistent with these studies we found that ⁵⁹Fe acquisition from NTA by HL-60 was inhibited by 90% in the presence of BPS and by 40% in the presence of FZ (Fig. 10). This inhibition of iron acquisition was observed with both gallium-induced and control cells. Inhibition of iron acquisition was observed in the presence of as little as 0.5 mM BPS with maximum inhibition seen at 1.5 mM BPS. The inhibitory effect of BPS on gallium-induced iron acquisition does not relate to the removal of cell-associated gallium. Cell-associated ⁶⁷Ga is virtually unaffected by subsequent incubation with 1 mM BPS.
In the acquisition process, ferricyanide might compete with the iron acquisition of HL-60 cells. If iron reduction were critical to the iron competitive substrate of membrane reductases (18), on the rate of K3Fe(CN)6 prior to measurement of iron acquisition (not co-incubated with [59Fe]NTA or with a pre-exposure of cells to FZ). However, BPS caused a 60% decrease in [67Ga]Ga(III) acquisition by induced cells (Fig. 11). This result implies that BPS interferes at least in part with iron acquisition by a mechanism which does not involve its interaction with reduced forms of iron.

As an alternative means of assessing the role of cellular reductase activity on iron acquisition by HL-60 cells, we examined the effect of potassium ferricyanide (K3Fe(CN)6), a competitive substrate of membrane reductases (18), on the rate of iron acquisition. If iron reduction were critical to the iron acquisition process, ferricyanide might compete with the Fe(III) chelate for the critical reductase (24, 26). However, we observed no inhibition of iron acquisition either when K3Fe(CN)6 was co-incubated with [59Fe]NTA or with a pre-exposure of cells to K3Fe(CN)6 prior to measurement of iron acquisition (not shown). In addition, we preincubated HL-60 cells with N-ethylmaleimide or p-chloromercuribenzenesulfonic acid, known chemical inactivators of membrane reductases. Neither of these reagents had any effect on iron acquisition or gallium induction. Together, these data along with the BPS/FZ effects raise substantial questions about the role of iron reduction in this acquisition process.

Is the Effect of Gallium-mediated Acquisition by Enhancement of Iron Reduction?—Given the ability of gallium to increase HL-60 cell iron acquisition from various chelates and some partial evidence (see above) that iron reduction could be involved in the iron acquisition process, we investigated the effect of gallium exposure on cellular reductase activity. Two separate techniques were employed for analyzing cellular reductase activity. First we measured the rate of cellular reduction of K3Fe(CN)6 (18) in the presence and absence of 1 mM Ga(NO3)3. We also measured the reduction of ferric iron bound to NTA by quantitating the formation of the characteristic Fe(II)-BPS chromophore which absorbs at 535 nm. The Fe(II)-BPS complex develops rapidly and is stable over several hours.

As shown in Table II and Fig. 12, although there was an increase in the reduction of iron in the presence of HL-60 cells, there was no further increase when these cells were treated with gallium. Thus, the mechanism by which gallium increases the rate of iron acquisition is not due to an increase in cell-mediated iron reduction.

Does Gallium Induction Involve Activation of Classical Signal Transduction Mechanisms?—Many modulations of myeloid cells involve signal transduction mechanisms mediated by protein kinase C (27). We investigated whether protein kinase C was involved in the effect of gallium on HL-60 iron acquisition by assessing the effect of both activators (phorbol 12-myristate 13-acetate) and inhibitors of these enzymes (staurosporine, H7) on HL-60 cells iron acquisition from NTA. Phorbol 12-myristate 13-acetate (100 ng/ml, 30 min) treatment had no effect on iron acquisition indicating that protein kinase C activation by itself was not sufficient to increase the rate of cellular iron acquisition. Furthermore, neither staurosporine (100 mM) nor H7 (50 mM) altered the rate of gallium induced iron acquisition when added just prior to gallium exposure or during the iron acquisition stage of the experiment. Tyrosine kinases have also been linked to key signal transduction processes in myeloid cells (28–31). However, genistein (300 μM, 1 h), herbimycin A (1 μg/ml, 24 h), and tyrphostin 23 (200 μM, 24 h), inhibitors of tyrosine kinases, did not alter gallium-induced HL-60 iron acquisition. Thus, these data do not suggest a role for protein kinase C or tyrosine kinases in either the gallium-induction or iron-acquisition process.

**DISCUSSION**

In earlier work we demonstrated that human monocytes, MDM, neutrophils and two myeloid cell lines (U937 and HL-60) possess a high capacity mechanism for the acquisition of iron from a variety of low molecular weight chelating agents (3, 14). The process could not be explained on the basis of standard mechanisms of cellular substrate acquisition (14). An important feature of the process was that the rate of iron acquisition...
TABLE II

| Treatment                  | Absorbance at 535 nm |
|---------------------------|----------------------|
| Background (no cells)     | 0.041 ± 0.018        |
| Cells only                | 0.121 ± 0.019        |
| Cells + Ga(NO₃)₃       | 0.108 ± 0.024        |
| Cells + ascorbate        | 2.972 ± 0.037        |

HL-60 cells were preincubated with Fe-NTA in the presence of 1 mM BPS (37 ºC). After 1 h, the cells were pelleted and absorbance of the supernatant quantitated at 535 nm. Shown are the results with no cells present (background), in the presence of non-gallium-treated cells (cells only), and with cells pretreated with gallium (cells + Ga(NO₃)₃). Also shown is the maximal potential iron reduction which was determined by the addition of 10 mM ascorbic acid to reduce all Fe³⁺ to Fe²⁺ (cells + ascorbate).

FIG. 12. Reductase activity is not affected by gallium induction of HL-60 cells. Cells were incubated with 1 mM K₃[Fe(CN)₆] without (□) or with (○) 30-min preexposure to 1 mM Ga(NO₃)₃ at 37 ºC. One ml of the cell suspension was withdrawn at varying time intervals and centrifuged (500 X g, 10 min). Reductase activity was monitored by change in the absorbance of the supernatant at 420 nm. Results shown are means ± S.D. of four separate experiments.

was markedly increased by preincubating the cells with iron, gallium, or aluminum. Previous work by others (12) had shown a similar effect of gallium in myeloid cells whereas iron had been shown to increase transferrin-independent iron acquisition in several non-myeloid cell types (12, 21, 22).

In spite of this evidence for a metal-mediated induction of transferrin-independent iron acquisition, the mechanism responsible has not been extensively studied. Using HL-60 cells as a model system, we extend our earlier observations (14) and now show that gallium markedly increases the rate at which HL-60 cells acquire iron from a variety of low molecular weight chelating agents. The gallium-induced cells appear to amplify their existing (base line) mechanism of transferrin-independent iron acquisition rather than activating a new system. Supporting this conclusion is the fact that the rate of gallium-induced iron acquisition varied with the nature of the chelate with the same hierarchy we previously noted with MDM in the absence of preincubation with trivalent metals (14). What physicochemical properties are responsible for the relative ability of different chelates to serve as iron donors is not known. Hypotheses are limited by a paucity of information on relative iron chelate affinities in physiologic solutions.

Trivalent Cation Association with the Cell Membrane—Iron acquired from gallium-treated or control cells is stably associated with the cell. In agreement with our earlier data using MDM (3), subcellular fraction analysis shows most of the acquired iron segregates with the plasma membrane containing fraction (Table I). The fate of the iron acquired by HL-60 cells in this study was not followed beyond 4 h. However, our previous MDM study found that, over 5 days in culture, membrane-associated iron moves from the membrane to cytosol, suggesting a gradual process of internalization. Numerous works by others (4, 5, 8, 13, 32–37) using various myeloid cell types are consistent with internalization of iron acquired from low molecular weight chelating agents.

Contrary to reports with other cells (12, 38), salts of the divalent metals cadmium, copper, manganese, or zinc had a minimal effect on iron acquisition from NTA or other chelating agents. Earlier data (14) suggesting that NTA chelates of these divalent metals inhibited iron acquisition were subsequently found to be the result of free NTA in the system. In contrast, aluminum, lanthanum, and gadolinium as well as the tetravalent metals tin or zirconium induced iron acquisition to a similar or greater extent than that observed with gallium (gadolinium ≈ lanthanum ≈ tin > gallium ≈ iron ≈ zirconium > aluminum). The polyvalent cation, polylysine, had no effect on HL-60 cell iron acquisition, indicating that the cell is not induced by just all multivalent cations. The activity of these metals could not be attributed to the anions to which they were initially complexed. Except for lanthanum, all metal cations with the ability to induce increased iron uptake in HL-60 have similar ionic radii ranging from 0.51–0.9 Å (39, 40). In contrast, divalent metal ions have ionic radii almost twice that of the trivalent metal ions, possibly providing some insight into the different potency of the divalent and trivalent metals for inducing iron acquisition.

Exposure to each of the trivalent metals was shown to result in a sustained increase in the rate of iron acquisition by the cells even if the metal was removed from the bulk solution. However, the rate of iron acquisition could be rapidly reversed to preinduction levels by subsequent exposure of the cells with NTA or citrate. The simplest explanation of these results is that the metal must remain associated with a critical cellular site in order to maintain the “induced” state. Another implication of these results is that the trivalent cation produces the induced state from a site accessible from the extracellular solution.

In addition, it seems possible that the variability of different iron chelates to cause the induced phenotype may be due to the differences in affinity of iron for the chelate relative to the cellular site leading to induction. As noted above, lack of definitive information about affinity for iron of many of these chelates in physiologic buffers limits assessment of this possibility.

Depleting HL-60 cells of ATP had no effect on the ability of gallium to increase the cells’ rate of iron acquisition. However, cells incubated at 4 ºC with gallium did not exhibit an “induced” rate of iron acquisition from NTA. These data suggest that if a metabolic response of the cell is required to produce the induced “phenotype,” that metabolic response does not appear to require the ability to generate ATP. Alternatively, the trivalent metals may mediate their effect by causing changes in the physical properties of the membrane (such as fluidity) which do not take place at low temperatures. The protein synthesis inhibitor cycloheximide also had no effect on the induction process, a result consistent with the rapidity with which induction occurs (30 min). The absence of a need for ATP or for protein synthesis in conjunction with the requirement that a trivalent cation be present on the extracellular surface of...
the cell raises the possibility that all of the elements necessary for this acquisition process are available on the cell membrane.

**Role of Reduction in Iron Acquisition**—Given evidence in other cells that iron reduction may be involved in iron acquisition processes (18, 24, 25), it seemed plausible that gallium and the other metals promoted iron acquisition by enhancing cellular reductase activity. However, using two separate detection techniques (Table II and Fig. 12) no such effect was detected. In addition, gallium markedly increases iron acquisition by HL-60 cells in the presence of ascorbate which will maintain all iron in its reduced form and thus negate the need for the presence of a reductase. These results imply that the iron and other trivalent metal-induced increases in HL-60 iron acquisition do not result from an increased rate of iron reduction.

Additional data raise questions as to the interpretation of experimental data from other cell types which suggest a role for iron reduction in transferrin-independent iron acquisition (18, 23–25). We observed that the ferrous iron chelators BPS and Ferrozine inhibited iron acquisition from NTA by both gallium-induced and control HL-60 cells, 90% and 40%, respectively. However, in spite of the fact that Ga^{3+} cannot readily be reduced to a divalent state, there was a 60% decrease in gallium acquisition by BPS. This result implies that at least part of the BPS interference with iron acquisition does not involve its interaction with reduced forms of that metal. For example, it could disturb cell membrane architecture through interaction with key membrane components. Furthermore, neither potassium ferricyanide, p-chloromercuribenzenesulfonic acid nor N-ethylmaleimide, known inhibitors of iron reductases (18, 24, 26), inhibited iron acquisition.

These data leave in some doubt that iron reduction is a critical component of myeloid cell acquisition of iron. Given the absence of data linking reduction of iron to its acquisition together with current and earlier data (12) that nonreducible trivalent cations (e.g. gallium) can be acquired by HL-60 cells in a similar manner we conclude that HL-60 cells possess an acquisition mechanism that is selective for trivalent metals.

**Signal Transduction and Induction**—Many modulations of myeloid cells involve signal transduction mechanisms mediated by protein kinase C (27). Our data do not suggest a role for protein kinase C in trivalent-mediated induction of iron acquisition in HL-60 cells. Neither a protein kinase C activator (phorbol 12-myristate 13-acetate) nor inhibitors (staurosporine and H7) had an effect on HL-60 cell iron acquisition. These studies appear to contrast earlier work with K562 cells in which an increase in iron acquisition was observed 18 h after exposure to a similar concentration of phorbol 12-myristate 13-acetate. However, the applicability of the K562 studies to the induction process we have examined is unclear given the differences in the time required (30 min versus 18 h) for the two effects. Furthermore, the features of the iron acquisition system of K562 cells in many ways differs from that described in other cells (37).

Aluminum increases iron acquisition from transferrin by Friend erythroleukemia cells (41) and increases membrane peroxidation (42) although this reportedly is not involved in uptake of non-transferrin bound iron by reticulocytes (43). A recent report concluded that hydroxyl radical production and its resultant effect on the cell membrane are responsible for the induction phenomenon in the SK-MEL-28 melanoma cell lines (44). It is unclear, however, how metals such as gallium which have not been reported to participate in free radical chemistry reactions could mediate their effects via this mechanism. Many of the inhibitors of membrane peroxidation employed in this work could have secondary effects on membrane integrity. Nevertheless, this is an intriguing set of observations which should be pursued.

**SUMMARY**

These results, taken together, describe previously unrecognized properties of a trivalent cation acquisition system. It can be induced by a variety of tri- and tetravalent cations; neither the induction nor the acquisition requires cellular metabolic energy; the magnitude of the acquisition and the induction processes are dependent on the specific anion (chelator); induction requires the presence of a (trivalent) cation on the cell surface; reductase activity is not required; and there is a large but finite capacity for iron acquisition. These characteristics/properties lead us to surmise that the cell membrane of myeloid cells, and perhaps other cells, possess physical properties uniquely suited for binding iron complexed to low molecular weight chelates. This iron acquisition system may play an important role in preventing toxic effects of iron.

**REFERENCES**