Resistance to the Antitumor Agent Gallium Nitrate in Human Leukemic Cells Is Associated with Decreased Gallium/Iron Uptake, Increased Activity of Iron Regulatory Protein-1, and Decreased Ferritin Production*

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The mechanism of drug resistance to gallium nitrate is not known. Since gallium can be incorporated into ferritin, an iron storage protein that protects cells from iron toxicity, we investigated whether ferritin expression was altered in gallium-resistant (R) CCRF-CEM cells. We found that the ferritin content of R cells was decreased, while heavy chain ferritin mRNA levels and iron regulatory protein-1 (IRP-1) RNA binding activity were increased. IRP-1 protein levels were similar in gallium-sensitive (S) and R cells, indicating that R cells contain a greater proportion of IRP-1 in a high affinity mRNA binding state. Gallium uptake and transferrin receptor expression were decreased in R cells. In both S and R cells, gallium inhibited cellular 59Fe uptake, increased ferritin mRNA and protein, and decreased IRP-1 binding activity. Gallium uptake by R cells was markedly diminished; however, the sensitivity of R cells to gallium could be restored by increasing their uptake of gallium with excess transferrin. Our results suggest that R cells have developed resistance to gallium by down-regulating their uptake of gallium. In parallel, iron uptake by R cells is also decreased, leading to changes in iron homeostasis. Furthermore, since gallium has divergent effects on iron uptake and ferritin synthesis, its action may also include a direct effect on ferritin mRNA induction and IRP-1 activity.

Gallium nitrate, a group IIIa metal salt with antineoplastic activity (1), is currently undergoing evaluation as a chemotherapeutic agent. A number of clinical studies have shown gallium to be effective in the treatment of lymphoma and bladder cancer (2–4). Although gallium is in clinical use, information regarding its action at the cellular and molecular levels is largely incomplete. It has been known for some time that gallium resembles iron in certain respects. Gallium binds avidly to the Tf receptor-dependent and -independent transport systems (6–8). Furthermore, we have been able to demonstrate the complete roads to cell lines with altered expression of the Tf receptor (9–11). Gallium nitrate is a group IIIa metal salt with antineoplastic activity (1), and is currently undergoing evaluation as a chemotherapeutic agent. A number of clinical studies have shown gallium to be effective in the treatment of lymphoma and bladder cancer (2–4). Although gallium is in clinical use, information regarding its action at the cellular and molecular levels is largely incomplete. It has been known for some time that gallium resembles iron in certain respects. Gallium binds avidly to the Tf receptor-dependent and -independent transport systems (6–8). Furthermore, we have been able to demonstrate the complete roads to cell lines with altered expression of the Tf receptor (9–11).
EXPERIMENTAL PROCEDURES

Materials—Gallium nitrate was obtained from Alpha Products (Danvers, MA). Heparin, bovine serum albumin, MTT, Tf, and dithiothreitol were obtained from Sigma. RNase T1 was obtained from Boehringer Mannheim. Na235, 35SFeCl3, 32P[GTP, and 32P[dCTP were purchased from Amersham Corp. 35S-Fe-Tf was prepared as described by Bates and Schlabach (33), and 32P-Tf was prepared by the chloramine-T method (34).

Cells—Human T lymphoblastic leukemia CCRF-CEM cells (S cells) were obtained from American Type Tissue Collection (Rockville, MD) and were grown in RPMI 1640 medium supplemented with 10% fetal calf serum in an atmosphere of 6% CO2 at 37 °C. A clone of CCRF-CEM cells resistant to the growth inhibitory effects of gallium nitrate (R cells) was developed in our laboratory through a process of continuous exposure of these cells to stepwise increments of gallium nitrate over the course of several months. R cells were routinely cultured in medium containing 150 μM gallium nitrate. For experiments, cells grown to confluency were harvested, washed with complete medium to remove gallium nitrate, and then subcultured in fresh medium in the presence or absence of gallium nitrate. At specified times, cells were harvested and analyzed as described below.

Cell Proliferation Assay—The effect of gallium on the proliferation of S and R cells was determined by MTT assay as described by Mossman (35). Cells were plated at an initial density of 2 × 104 cells/ml in 96-well microplate wells and incubated for 72 h in the presence of 0–1000 μM gallium nitrate. At the end of the incubation, 10 μl of MTT (5 mg/ml stock solution) was added to each well, and the cells were incubated at 37 °C for an additional 4 h. Cells were then solubilized with the addition of 100 μl of 0.1 N HCl in isopropanol alcohol to each well, and the absorbance of each well was determined spectrophotometrically at a dual wavelength of 570/630 nm using an EL 310 microplate auto-reader (Biotek Instruments, Winooski, VT). The effect of gallium nitrate on cell proliferation was determined by comparing the absorbance of the wells containing gallium nitrate with that of wells in which the drug was omitted. In some experiments, cell number was determined by direct counting with a hemocytometer.

cDNA Probes and Antisense—cDNAs for rat L and H ferritin cloned into pGEM4Z and pSP65 vectors (Promega, Madison, WI), respectively, and rabbit antisem to IRP-1 were generously provided by Richard Eisenstein and have been previously described (36). cDNAs for L and H ferritin were excised from the plasmids with PstI and EcoRI, respectively, and were 32P-labeled using a RadPrime DNA labeling system from Life Technologies, Inc. Human β-actin cDNA probe was obtained from CLONTECH (Palo Alto, CA). 32P-Labeled IRE mRNA for the RNA band-shift assay was prepared, using as a template, a 1000-base pair rat L ferritin pseudogene that contains the conserved IRE sequence. The plasmid (p66-L gene) containing this insert (generously provided by Elizabeth Leibold) (13) was linearized with Smal (Life Technologies, Inc.) and used for in vitro transcription of IRE mRNA. Transcription was carried out with SP6 RNA polymerase using a Riboprobe transcription system from Promega.

Measurement of Cellular Ferritin—Ferritin in S and R CCRF-CEM cells was measured before and after a 24-h incubation in fresh medium. Cells were harvested; washed with 10 mM KPO4, pH 7.4, 150 mM NaCl buffer (PBS); and disrupted by sonication. Cellular debris was removed by centrifugation (30,000 × g for 30 min), and the cytoplasmic fraction (supernatant) was assayed for protein content using a Pierce BCA protein assay and for total ferritin using a Bio-Rad Quantamune ferritin assay. The ferritin content is expressed as ng/mg of protein.

RNA Isolation and Northern Blotting—Total cellular RNA was isolated from S and R cells by a modification of the method of Chomczynski and Sacchi (37) using RNAzol (Tel-Test Inc., Friendswood, TX) according to the manufacturer's recommendations. The integrity of the RNA was verified by agarose gel electrophoresis. Twenty micrograms of RNA from each preparation was electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde and transferred to Nytran membranes (Schleicher & Schuell) using the capillary-blotting method. H and L ferritin and β-actin mRNA were detected by sequencing hybridization of the membranes to the corresponding 32P-labeled cDNA probes (1.2 × 106 cpm/mg of probe), and the intensity of the RNA bands was quantitated by scanning the autoradiograms with a phosphorimager (Molecular Dynamics). The equal loading of RNA on the gels and to normalize the results obtained with hybridization using 32P-labeled L and H ferritin cDNA probes.

RNA Band-shift Assay—The binding of IRP-1 to ferritin IRE mRNA was examined by an RNA band-shift assay as described by Leibold and Munro (13). For preparation of cytoplasmic extracts, S and R cells were incubated for 24 h in fresh medium with or without gallium. Cells were then harvested by centrifugation (3500 × g for 10 min) and were washed twice with ice-cold PBS and 4 mM HEPES, pH 7.6, containing 5% glycerol, 0.5 mM EDTA, 25 mM KCl, 1 mM dithiothreitol, 1% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride. Cell lysates were centrifuged at 30,000 × g for 30 min, and the protein content of the supernatant was measured. For the band-shift assay, 40 μg of cytoplasmic extract from cells was incubated with 200,000 cpm of 32P-labeled IRE mRNA in binding buffer containing 10 mM HEPES, pH 7.6, 3.0 mM MgCl2, 40 mM NaCl, 1 mM dithiothreitol. To specifically identify IRP-1 binding to the IRE, incubation conditions were as described above, except that cytoplasmic extracts were incubated in binding buffer with 5 μl of antiserum to IRP-1 for 1 h at 4°C prior to the addition of 32P-labeled IRE mRNA. After a 30-min incubation at room temperature, 1 unit of RNase T1 was added to the reaction mixture, and incubation was continued for an additional 30 min. Heating the reaction mixtures at 80°C for 10 min was used to inactivate RNase T1.

Ferritin in S and R CCRF-CEM cells was determined after these cells had been washed to remove gallium and were grown in gallium-containing culture medium. 59Fe uptake by R cells was also determined after these cells had been washed to remove gallium and cultured for 72 h in culture medium without additives.

Gallium Uptake Studies—To measure the cellular uptake of gallium, cells were plated in triplicate in fresh medium (5 × 104 cells/ml) in 1-mL liftwell plates. Gallium nitrate (100–1000 μM) containing 125I-Ga as a tracer (1 μCl of 125I(Ga-1 μM gallium nitrate) was added to wells at the onset of incubation. After 48 and 72 h of incubation, cell counts were determined, and the cells were harvested and washed by centrifugation with PBS. 67Ga radioactivity in the cell pellet was counted in a Wallac Compugamma γ-counter, and the amount of 67Ga incorporated per 106 cells was determined.

125I-Tf Binding Studies—Cell-surface Tf receptor density was determined by 125I-Tf binding to intact cells. Confluent S and R CCRF-CEM cells were washed and resuspended in fresh medium without gallium nitrate. After 24 and 48 h of incubation, cells were harvested, washed with ice-cold PBS containing 0.1% bovine serum albumin, and assayed for 125I-Tf binding at 37°C as described previously (40). In separate experiments, R cells that had been grown for 8 weeks in the absence of gallium were also assayed for 125I-Tf binding. Maximal Tf binding and Tf receptor affinity for Tf were determined according to the method of Scatchard (41).
incubation of cells with gallium induced a marked increase in H and L ferritin mRNAs in both R and S cells (Fig. 2, upper panel). As illustrated further in the lower panel of Fig. 2, H ferritin mRNA levels in S cells increased in a dose-dependent manner following exposure to increasing concentrations of gallium. S cells incubated with 50 and 150 μM gallium nitrate displayed 1.6- and 3.7-fold increases in H ferritin mRNA, respectively, relative to cells grown without gallium.

**IRP-1 Activity Is Increased in Gallium-resistant Cells**—The above experiments suggested that although H and L ferritin mRNA levels were increased in R cells, their translation was inhibited. Since ferritin synthesis is regulated at the translational level by the binding of IRPs to H and L ferritin mRNAs, IRP binding activity in S and R cells was examined by band-shift assay. As shown in Fig. 3, IRP mRNA binding was increased 2.4-fold in R cells incubated for 24 h in the absence of gallium (compare lanes 3 and 4 with lanes 1 and 2). Antibody to IRP-1 produced a supershift in these bands (lanes 9 and 10), indicating that they specifically represent IRP-1 binding to ferritin mRNA. Fig. 3 also shows that in the presence of gallium, IRP activity in both S and R cells was decreased; however, it still remained greater in R cells than in S cells.

To exclude the possibility that the differences in IRP-1 activity in R cells were due to changes in the amount of IRP-1 protein, cells were analyzed by Western blotting after a 24-h incubation with increasing concentrations of gallium nitrate (0–250 μM). In three separate experiments, S and R cells were found to contain comparable amounts of IRP-1, which did not change significantly following exposure of cells to gallium (data not shown).

**Iron Uptake by Gallium-sensitive and -resistant CCRF-CEM Cells**—Since prior studies showed that gallium inhibits the cellular uptake of iron (9), we investigated whether the differences in ferritin content between S and R cells could be explained by an effect of gallium on iron transport into cells. Cells were therefore examined for 59Fe uptake in the presence and absence of gallium nitrate. As shown in Fig. 4, 59Fe uptake by S cells was decreased by ~31% in the presence of 150 μM gallium nitrate. In contrast, 59Fe uptake by R cells was markedly decreased even in the absence of gallium nitrate and was ~60% lower than that by S cells. With the addition of gallium nitrate to the incubation, 59Fe uptake by R cells was decreased further, albeit to a lesser extent than that seen with S cells.

Prior to being used in these experiments, R cells were washed extensively to remove gallium present in the stock cultures; however, to confirm that the decrease in iron uptake by R cells was not due to residual gallium in the system, 59Fe uptake studies were also performed using R cells that had washed, incubated for 3 days in fresh medium without gallium, and then washed again. 59Fe uptake by these cells (R1 cells) was identical to that seen with washed R cells taken directly from stock cultures (Fig. 4). Even after 12 days (three passages)
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Fig. 2. Northern blot analysis. Upper panel, comparison of H and L ferritin mRNAs in gallium-sensitive and -resistant CCRF-CEM cells. S and R cells were incubated for 24 h in the presence or absence of gallium nitrate. Total cellular RNA was isolated, electrophoresed on a formaldehyde-agarose (1%) gel, and transferred to a membrane. The membrane was hybridized sequentially to 32P-labeled cDNAs for H ferritin, L ferritin, and β-actin. The membrane was stripped of the respective 32P-labeled cDNA probes between each hybridization. Data shown are from a single membrane and are representative of six separate experiments. Lower panel, effect of gallium on H ferritin mRNA levels in gallium-sensitive cells. Cells were incubated for 24 h in the presence or absence of gallium nitrate. Total cellular RNA was isolated, electrophoresed on a formaldehyde-agarose (1%) gel, and transferred to a membrane. The membrane was hybridized to 32P-labeled cDNA for H ferritin. Equal RNA loading in each well was confirmed by hybridization of the membrane to 32P-labeled β-actin (not shown). Data shown are representative of three separate experiments.

Gallium Transport into Resistant Cells Is Decreased—To determine whether the mechanism of drug resistance to gallium involves changes in its transport into cells, gallium uptake by R cells was compared with that of S cells. To correlate gallium transport into cells with the cytotoxicity assays, gallium uptake by cells was examined after 48- and 72-h incubations in the presence of increasing concentrations of gallium nitrate with 67Ga as a tracer. When incubated with 100 μM gallium (a nontoxic concentration), both S and R cells incorporated similar amounts of gallium/cell over 48 and 72 h (Fig. 5). Incubation with higher concentrations of gallium nitrate resulted in increases in gallium uptake by both S and R cells; however, gallium incorporation into R cells was significantly lower and, in the presence of 1000 μM gallium nitrate, was only 34–36% that into S cells (Fig. 5).

125I-Tf Binding Studies—125I-Tf binding to S and R cells was examined to determine whether the decrease in gallium and iron uptake by R cells could be explained on the basis of a decrease in Tf receptors. As shown in Fig. 6A, 125I-Tf binding to R cells was 72 and 54% that of S cells after 24 and 48 h of incubation, respectively, in the absence of gallium. To examine whether this decrease in Tf receptors was related to a residual effect of exogenous gallium, 125I-Tf binding to R cells was measured again after 8 weeks of growth of these cells in medium without gallium. Drug resistance to gallium was retained even after growth of these cells in the absence of gallium (data not shown). Scatchard analysis of 125I-Tf binding to cell-surface Tf receptors (shown in Fig. 6B) revealed that maximal 125I-Tf binding to R cells was ~53% that of S cells at 48 h without a significant change in Tf receptor affinity for Tf (Kd = 9.8 × 10−9 M for R cells).

Fig. 3. IRP binding activity in gallium-sensitive and -resistant cells. Cells were analyzed for IRP-1 mRNA binding activity after 24 h of incubation with or without 250 μg gallium as detailed under “Experimental Procedures.” Upper panel, band-shift assay. Lanes 1 and 2, S cells incubated without gallium; lanes 3 and 4, R cells incubated without gallium; lanes 5 and 6, S cells incubated with gallium; lanes 7 and 8, R cells incubated with gallium; lane 9, S cells incubated without gallium and supershifted with IRP-1 antibody; lane 10, R cells incubated without gallium and supershifted with IRP-1 antibody. Lower panel, relative IRP binding activity. Band intensities of lanes 1–8 were determined by densitometry. Bars represent the mean band intensities of duplicate lanes and are relative to S cells incubated without gallium.

Fig. 4. 59Fe uptake. S and R cells were washed with medium and plated (5 × 104 cells/ml) with (+) or without (−) 150 μM gallium nitrate. 59Fe-Tf was added at the onset of incubation, and 59Fe uptake by cells was determined after 24 h of growth. Resistant/R1 cells represent R cells that had been grown in fresh medium without gallium nitrate for 72 h prior to the 59Fe uptake study. Bars represent mean ± S.E. (n = 3).

Transferrin Increases Gallium Uptake by R Cells and Restores Sensitivity to Gallium—The above experiments show that R cells have a decrease in gallium uptake and changes in transferrin receptor number. Since Tf is known to enhance gallium uptake by cells (6, 7, 9), further experiments were performed to determine whether increasing the amount of exogenous Tf would enhance gallium uptake and increase its cytotoxicity in R cells. As shown in Fig. 7, the presence of 1 mg/ml transferrin resulted in a marked increase in the uptake...
of gallium by R cells (upper panel) and a progressive inhibition of cell proliferation (lower panel). When added to R cells incubated with 1000 \(\mu\text{M}\) gallium, Tf produced a 3.4-fold increase in gallium uptake and completely inhibited cell proliferation.

DISCUSSION

As an iron storage protein, ferritin plays an important role in protecting cells from the toxicity of excessive intracellular iron. In addition to sequestering iron, however, ferritin can also bind gallium (7, 32). This latter property of ferritin prompted us to initially investigate whether the development of drug resistance to gallium was associated with changes in ferritin gene expression. Our studies revealed that whereas ferritin mRNA levels were increased in R cells, ferritin protein content was markedly diminished. Further investigation demonstrated that IRP-1 RNA binding activity was increased, thus suggesting that the decrease in ferritin production in R cells was the result of a repression of ferritin mRNA translation. IRP-1 protein levels were equivalent in R and S cells, indicating that the increase in IRP-1 binding activity in R cells was due to a greater proportion of IRP-1 existing in a high affinity mRNA binding state. Since IRP/RNA interactions are directly influenced by changes in cellular iron, iron uptake studies were carried out that showed that \(^{59}\text{Fe}\) incorporation into R cells was <50% that into S cells even in the absence of gallium. Hence, it appears that the switch in IRP-1 from a low to a high affinity mRNA binding state in R cells is due to a down-regulation of iron uptake and a depletion of an intracellular iron “pool” responsible for influencing IRP binding activity.

Whereas the inhibitory effect of gallium on iron uptake by S and R CCRF-CEM cells was consistent with earlier results in other gallium-sensitive cell lines (9), an unexpected finding was that iron uptake by R cells was consistently less than that by S cells even after extended growth of R cells in the absence of gallium. Since earlier studies have shown that cells incorporate iron and gallium by similar Tf-dependent and -independent transport systems (6–8), we questioned whether the uptake of gallium by R cells was also decreased. Gallium uptake studies were therefore performed that showed that when challenged with increasing concentrations of gallium nitrate, R cells incorporated significantly less gallium than S cells. Hence, it appears that R cells have a decrease in the activity of a metal uptake transport system that affects both gallium and iron. This decrease in gallium uptake would serve to protect cells from the cytotoxicity of gallium, while the parallel decrease in iron uptake would lead to changes in intracellular iron homeostasis.

While the gallium uptake studies strongly suggest that the
presence of increasing concentrations of gallium nitrate secondary to the decrease in iron uptake, the effect of gallium in resistant cells. Cells were plated at 0.5 x 10^6/ml in the presence of increasing concentrations of gallium nitrate containing 67Ga (1 μCi/ml) as a tracer. Gallium uptake (67Ga) was determined after 72 h in the absence (upper panel, left) and presence (upper panel, right) of transferrin (Tf). Corresponding cell counts are also shown (lower panel).

Development of gallium resistance is primarily due to a down-regulation of gallium transport into cells, the specific transport mechanisms involved remain to be fully elucidated. One explanation for the decrease in gallium uptake is that the lower number of Tf receptors expressed on R cells during proliferation may serve to limit transferrin-mediated uptake of Tf-gallium complexes (formed by the binding of gallium to Tf in the culture medium). Alternatively, the decrease in gallium uptake by R cells may be secondary to a decrease in the activity of a transferrin-independent gallium/iron transport system. It is conceivable that both transport systems work in concert to decrease the uptake of gallium by R cells. Regardless of the membrane transport system involved, our studies indicate that the decrease in gallium uptake is central to the mechanism of drug resistance to gallium. Further evidence for this mechanism is provided by the demonstration that the sensitivity of R cells to gallium could be restored by increasing their uptake of gallium with excess Tf (Fig. 7). Increasing the amount of Tf favors the formation of Tf-gallium complexes and stimulates Tf receptor-mediated uptake of gallium, thereby overriding the basal decrease in gallium transport into R cells. Studies are in progress to elucidate how Tf-dependent and -independent gallium transport pathways are regulated in R cells and to determine their relative roles in gallium resistance.

Although the decrease in ferritin synthesis in R cells appears to be secondary to the decrease in iron uptake, the effect of gallium on ferritin gene expression appears to be complex. Since gallium inhibited iron uptake by both S and R cells, cells incubated with gallium would be expected to contain less ferritin than cells incubated without gallium. Instead, cells incubated with gallium contained more ferritin mRNA, lower IRP-1 binding activity, and more ferritin protein than cells incubated without this metal. These results suggest that gallium may affect ferritin expression through mechanisms that are independent of its inhibitory action on cellular iron uptake. Although there is no evidence that gallium interacts directly with IRP-1 to alter its mRNA binding activity, Cowley et al. (42) have reported the synthesis of a cubic gallium-sulfur cluster with gallium in place of iron. This raises the intriguing possibility that intracellular gallium may be capable of interacting with the iron-sulfur cluster of IRP-1 to produce a change in its affinity for the IREs. However, an alternative explanation for the gallium-induced increase in ferritin may be that intracellular gallium displaces iron from its binding to different ligands/macromolecules, thereby leading to an increase in an iron pool, which, in turn, produces a decrease in IRP activity and an increase in ferritin synthesis.

While a direct interaction of gallium with IRP-1 remains speculative at this time, our studies clearly demonstrate that gallium, in a dose-dependent manner, increases H ferritin mRNA and, to a lesser degree, L ferritin mRNA. The mechanism for this increase is unknown and is under investigation. Several studies have shown that ferritin gene expression can be induced by a number of stimuli that are unrelated to iron. Cytokines such as interleukin-1α, tumor necrosis factor-α, and interferon-γ induce H ferritin mRNA transcription and increase H ferritin synthesis in cells (43, 44), whereas interleukin-1β increases the synthesis of both H and L ferritin by increasing mRNA translation (45). More recently, thyroid hormone has been shown to increase ferritin synthesis by modulating the interaction between IRP and ferritin mRNA (46). Hence, it is conceivable that gallium may influence ferritin mRNA transcription and IRP activity through mechanisms that are independent of iron metabolism.

In conclusion, these studies are the first to show that the development of drug resistance to gallium involves a diminution in the transport of gallium into cells and that this alteration in gallium transport also affects iron transport and ferritin gene expression. Continued investigation of the interaction of gallium with biological systems may enhance our understanding of the mechanisms of its transport, cytotoxicity, and drug resistance and may allow us to increase its efficacy as an antineoplastic agent.

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J. Biol. Chem. 1997, 272:12151-12157.
doi: 10.1074/jbc.272.18.12151

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