Development of Drug Resistance to Gallium Nitrate through Modulation of Cellular Iron Uptake

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ABSTRACT
We have shown that transferrin-gallium (TF-Ga) blocks DNA synthesis through inhibition of cellular iron incorporation and a diminution in the activity of the iron-dependent M2 subunit of ribonucleotide reductase. To examine the mechanisms of drug resistance to gallium, we developed a subline of HL60 cells (R cells) which is 29-fold more resistant to growth inhibition by gallium nitrate than the parent line (S cells). R cells displayed a 2.5-fold increase in transferrin (TF) receptor expression, without a change in receptor affinity for TF. The uptake and release of $^{67}$Ga were similar for both S and R cells. The uptake of $^{59}$Fe-Tf by S cells was inhibited by gallium nitrate over 24–48 h of incubation. In contrast, $^{59}$Fe-Tf uptake by R cells, although initially inhibited by gallium nitrate at 24 h, was no longer inhibited at 48 h of incubation. $^{59}$FeCl$_3$ uptake by R cells was significantly greater than that of S cells, regardless of the time in culture. Despite the increase in $^{59}$Fe uptake by R cells, the ferritin content of these cells was lower than that of S cells. The ribonucleotide reductase electron spin resonance signal of R cells was comparable to that of S cells. R cells were not cross-resistant to Adriamycin, vincristine, cis-platinum or hydroxyurea. Resistance to gallium nitrate in this subline of HL60 cells results primarily from the ability of cells to overcome the gallium-induced block in iron incorporation. In addition, intracellular iron in R cells appears to traffic preferentially to a non-ferritin compartment.

INTRODUCTION
Gallium, a group 11A metal, is currently undergoing clinical investigation as a chemotherapeutic agent for the treatment of certain malignancies (gallium nitrate, NSC 15200) (1). While the antitumor activity of this agent has been documented for some time, the mechanism of cytotoxicity of gallium has only recently been elucidated (2, 3). The avid binding of gallium to the iron transport protein Tf$^+$ results in the formation of stable TF-Ga complexes (4) which are incorporated into cells through TF receptor-mediated endocytosis (5, 6). We have shown that exposure of HL60 cells to TF-Ga results in a decrease in iron uptake, an increase in cellular TF receptor number (resulting from intracellular iron deficiency), and a subsequent arrest in the growth of these cells (7). The inhibition of cellular proliferation is the result of a diminution in the activity of the iron-dependent M2 subunit of ribonucleotide reductase (2). This enzyme is responsible for the reduction of ribonucleotides to deoxyribonucleotides, a rate-limiting step in DNA synthesis (8). The inhibitory effects of gallium on cell growth can be reversed by iron salts (9), TF-Fe (7), hemin (2), or iron-pyridoxal isonicotinoylhydrazone (10).

The development of resistance to chemotherapeutic agents by tumor cells remains a major obstacle to the successful treatment of malignancy. To understand how cells may overcome the cytotoxicity of gallium, we have developed a subline of HL60 cells which is relatively resistant to growth inhibition by this metal. We have found that the primary mechanism of drug resistance to gallium nitrate involves the ability of cells to overcome the gallium-induced block in iron uptake. As a result, gallium-resistant cells are able to acquire sufficient iron for DNA synthesis.

MATERIALS AND METHODS

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The abbreviations used are: Tf, transferrin; TF-Ga, transferrin-gallium; S cells, gallium-sensitive cells; R cells, gallium nitrate-resistant cells; ESR, electron spin resonance; PBS, phosphate-buffered saline.

Chemicals. Gallium nitrate and human Tf were obtained from Alfa Products (Danvers, MA) and Sigma Chemical Co. (St. Louis, MO), respectively. Sodium $^{125}$Iodide and $^{59}$FeCl$_3$ were obtained from New England Nuclear (Boston, MA). Saturation of TF with $^{59}$Fe and iodination of $^{38}$Fe-Tf were performed as described previously (11). Hydroxyurea and vincristine were obtained from Calbiochem-Behring Corp. (San Diego, CA) and LyphoMed, Inc. (Rosemont, IL), respectively. Adriamycin and cis-platinum were purchased from Adria Laboratories (Columbus, OH) and Bristol Laboratories (Bristol, NY), respectively.

Cells and Cell Growth Studies. Human promyelocytic leukemic HL60 cells were obtained from American Type Culture Collection (Rockville, MD) and were maintained in RPMI 1640 containing 10% fetal calf serum with 200 units/ml of penicillin, 0.2 mg/ml of streptomycin, and 50 µg/ml of gentamicin (complete medium). A gallium nitrate-resistant HL60 subline (R cells) was developed over a period of 9–12 months by growing cells in increasing concentrations of gallium nitrate. In vitro, the concentration of gallium nitrate in the medium was made only when the growth of R cells was equivalent to that of gallium nitrate-sensitive cells (S cells). Once a resistant subline had been established, R cells were routinely subcultured with 84 µM gallium nitrate in the medium. For studies of growth kinetics, confluent cells were plated at 5 × 10$^4$ cells/ml in multiwell plates in the presence of varying concentrations of gallium nitrate, Adriamycin (0.01–1.0 µM), vincristine (1–10 nM), cis-platinum (0.5–2.5 µM) or hydroxyurea (25–300 µM). Cell counts were performed after 72 h of incubation and cell viability was determined by trypan blue exclusion.

$^{125}$I-Tf Binding Studies. Cell surface Tf receptor density was determined by $^{125}$I-Tf binding to intact cells as described previously (11). S and R HL60 cells plated with or without 84 µM gallium nitrate were harvested after 24, 48, and 72 h of incubation. Cell counts at these time points were approximately 0.8, 1.4, and 2.2 × 10$^5$/ml, respectively.

$^{59}$Fe Uptake and Release Studies. Confluent S and R cells were replated in complete medium (5 × 10$^4$ cells) in 1-ml multiwell plates. $^{67}$Ga citrate, 0.5 µCi, was added to each culture. After 24 and 48 h of incubation, cells were harvested by centrifugation and the radioactivity in cell pellets was counted to determine the amount of $^{67}$Ga incorporated by cells. To examine the release of $^{67}$Ga from cells, R and S cells which had incorporated $^{67}$Ga over 24 or 48 h were washed twice and reincubated at 37°C in fresh medium. Aliquots of cell suspension were removed after 2, 4, 6, and 24 h of incubation and centrifuged (2000 rpm for 10 min). The radioactivity in the cell pellet and supernatant was counted to determine the fraction of $^{67}$Ga released from cells over time.

$^{59}$Fe Uptake Studies. Confluent S and R cells were replated (5 × 10$^5$ cells) in 1-ml multiwell plates in the absence or presence of 84 µM...
gallium nitrate. $^{59}$Fe-Tf (approximately 2 µg Tf/ml) was added to each well and cells were harvested after 24 and 48 h of incubation at 37°C in a CO2 incubator. Cells were washed with PBS by centrifugation at 1000 rpm for 10 min and the radioactivity in the cell pellets was counted. In a separate experiment, S and R cells which had been subcultured for 0, 24, or 48 h in their respective media (without or with 84 µM gallium nitrate) were harvested, washed, and reincubated in corresponding fresh media (5 × 10⁶ cells/ml) with $^{59}$FeCl₃ (10 ng $^{59}$Fe, 115,000 cpm/ml) for an additional 24 h. Cells were harvested by centrifugation and the radioactivity in the cell pellet was counted.

Measurement of Cellular Ferritin Content. S and R cells were harvested, washed twice with PBS containing 0.1% bovine serum albumin and were resuspended in the same buffer containing 0.1% Triton X-100. After disruption of cells by sonication, cellular debris was removed by centrifugation (10,000 × g for 30 min) and the cytoplasmic supernatant was assayed for ferritin using a radioimmunoassay kit from BioRad (Richmond, CA).

ESR Spectroscopy Studies. Studies of the tyrosyl free radical of ribonucleotide reductase were performed on HL60 cells as previously described by us (2). Approximately 5 × 10⁸ S and R cells were incubated for 24 h in medium containing 0-960 µM gallium nitrate. Cells were harvested and washed with PBS and direct ESR measurements were performed on frozen samples at −196°C in quartz finger Dewar flasks. ESR spectra were recorded nine times and averaged by computer.

RESULTS

Growth of S and R Cells in the Presence of Gallium Nitrate. The dose-response curves of S and R cells to gallium nitrate are shown in Fig. 1. The concentration of gallium nitrate required to inhibit cell growth by 50% was 79.6 µM for the S cells and 2295.8 µM for the R cells. Hence, the R cells were approximately 29 times more resistant to growth inhibition by gallium nitrate than the S cells.

Transferrin Receptor Expression. In previous studies, we have shown that the uptake of $^{67}$Ga by HL60 cells is mediated by the binding of $^{65}$Ga-Tf complexes to cellular Tf receptors and that the uptake of this metal closely correlates with the density of cell surface Tf receptors (6). Therefore, to determine whether R cells expressed altered numbers of Tf receptors, cells were assayed for Tf binding following various times in subculture. As shown in Table 1, at 24 h R cells in gallium nitrate expressed a markedly greater number of Tf receptors than S cells exposed to the same concentration of gallium. At 48 h, R cells were compared with S cells plated without gallium, so as to examine Tf receptor expression under conditions of equal growth. As shown in Fig. 2, R cells displayed almost twice the number of Tf receptors as S cells, without a change in receptor affinity for the ligand. At 72 h of subculture, when cells had attained confluency, Tf receptor expression on R cells was approximately 2.5 times that of S cells (Fig. 2, inset).

$^{65}$Ga Uptake by Cells. To examine whether resistance to 65Ga uptake by S cells could be explained on the basis of differences in the cellular incorporation of gallium, $^{65}$Ga by R and S cells was compared (Table 2). The $^{65}$Ga cpm incorporated by S cells at 24 and 48 h was not significantly different from the $^{65}$Ga cpm incorporated by R cells at the same time points (P = 0.14 and 0.5 by 2-tailed unpaired t test for the 24- and 48-h time points, respectively). Furthermore, although $^{65}$Ga uptake by S cells did increase from 24 h to 48 h, this increase failed to reach statistical significance (P = 0.063). Likewise, the increase in $^{65}$Ga uptake by R cells from 24 h to 48 h was not significant (P = 0.46). In additional experiments, cells which had incorporated $^{65}$Ga over 24 or 48 h were washed and allowed to release $^{65}$Ga in fresh medium. No differences were noted in the release of $^{65}$Ga from R and S cells (not shown).

$^{59}$Fe Uptake by Cells. We have shown previously that the growth-inhibitory effects of Tf-Ga result from an inhibition of
GALLIUM RESISTANCE AND IRON UPTAKE

Iron incorporation into HL60 cells (7). Incubation of S cells with 84 μM gallium nitrate resulted in a marked inhibition of 59Fe uptake (as 59Fe-Tf) at 24 and 48 h of incubation (Fig. 3A). This decrease in iron uptake resulted in an arrest in the proliferation of these cells. As shown in Fig. 3B, 59Fe uptake by R cells at 24 h was also inhibited by 84 μM gallium nitrate. However, at 48 h, 59Fe uptake by these cells was no longer inhibited by gallium nitrate and as a result, R cells proliferated normally.

As stated previously, resistance to gallium was developed through routine growth of R cells in the presence of gallium nitrate in medium supplemented with 10% fetal calf serum (containing bovine Tf). To exclude any influence that exogenously added human Tf may have had on the uptake of 59Fe in the above studies, S and R cells growing in their usual culture medium were pulsed with 59FeCl3 at different times of subculture. As shown in Fig. 4, the uptake of 59Fe from 59FeCl3 was always greater in the R cells regardless of the time points examined. The 59Fe uptake studies suggest strongly that R cells overcome the growth-inhibitory effects of gallium by a sustained up-regulation of the Tf receptor coupled with an increase in the incorporation of Fe.

Cellular Ferritin Content. Table 3 shows that regardless of the time in subculture, the ferritin content of R cells grown continuously in the presence of 84 μM gallium nitrate was markedly lower than that of S cells. From the data shown in Figs. 3 and 4, it should be appreciated that R cells contained less ferritin than S cells despite the fact that the uptake of 59Fe (from either 59Fe-Tf or 59FeCl3) was greater in R cells.

Effects of Gallium Nitrate on the Tyrosyl Free Radical of Ribonucleotide Reductase. Ribonucleotide reductase consists of an effector-binding M1 subunit and an iron-containing M2 subunit (8). The M2 subunit also contains a tyrosyl free radical which produces a characteristic signal on ESR spectroscopy, and the activity of this subunit correlates closely with the magnitude of this ESR signal (13, 14). Exposure of HL60 cells to Tf-Ga results in a marked decrease in the amplitude of this ESR signal and a decrease in deoxyribonucleotide synthesis (2). As shown in Fig. 5, the ESR spectra of S and R cells were examined following incubation of cells with increasing concentrations of gallium nitrate. Consistent with our earlier studies (2), increasing concentrations of gallium nitrate resulted in a marked suppression in the ESR signal of S cells. In contrast, this effect was not seen with R cells. In the absence of gallium, the ESR signals of S and R cells were of comparable magnitude, thereby suggesting that under conditions of equal cellular proliferation, the activity of the M2 subunit of ribonucleotide reductase was similar in both cell populations.

Lack of Cross-Resistance between Gallium Nitrate and Other Antineoplastic Agents. The sensitivities of S and R cells to Adriamycin, vincristine, cis-platinum, and hydroxyurea were compared. Drug resistance to Adriamycin and vincristine (multidrug resistance) is mediated by a cell membrane-based P-glycoprotein which facilitates drug efflux from cells (15). The metal cis-platinum has been reported to bind to Tf (16), and resistance to this drug is related to an overexpression of metallothionein (17). Mouse L-cells have been shown to develop resistance to hydroxyurea through an increase in the M2 subunit of ribonucleotide reductase resulting from gene amplification (18). No differences in the sensitivity of R and S cells to the above antineoplastic agents were seen (data not shown). These studies indicate that the mechanism of resistance to cell growth inhibition by gallium is unique and is independent of other known mechanisms of drug resistance.

DISCUSSION

Although the mechanisms of tumor cell resistance to many drugs are not known, recent studies have defined the basis of drug resistance to certain antineoplastic agents. These mechanisms include decreased drug transport into the cell, increased drug efflux from the cell, alterations in or overproduction of intracellular target proteins, gene amplification, and increased drug degradation (19). Our present study describes an addi-

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<th>Time in subculture (h)</th>
<th>Ferritin content (μg/mg protein)</th>
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<tbody>
<tr>
<td>24</td>
<td>Sensitive: 18.2 ± 3.8</td>
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<tr>
<td></td>
<td>Resistant: 5.0 ± 0.5</td>
</tr>
<tr>
<td>48</td>
<td>Sensitive: 17.6 ± 1.1</td>
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<tr>
<td></td>
<td>Resistant: 5.7 ± 0.3</td>
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<tr>
<td>72</td>
<td>Sensitive: 20.0 ± 2.7</td>
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<tr>
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<td>Resistant: 6.0 ± 0.6</td>
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Fig. 5. ESR spectra of the tyrosyl free radical of ribonucleotide reducense. Gallium nitrate-sensitive (S) and -resistant (R) HL60 cells were harvested after 24 h of exposure to 0-960 μM gallium nitrate. The ESR spectra of gallium nitrate-sensitive (S) and -resistant (R) cells are shown in the left and right columns, respectively. Gallium nitrate (GN) concentrations are shown in the middle column. ESR measurements were performed at ~196°C on frozen samples containing 5 x 10^6 cells. Spectrometer conditions: microwave frequency, 9.098 GHz; modulation amplitude, 5 Gauss; modulation frequency, 100 kHz; incident microwave power, 100 mW; gain, 8 x 10^4; and temperature, ~196°C. Data represent the signal average of nine scans.

Functional mechanism in which drug resistance is mediated by the combination of sustained up-regulation of a cellular receptor and enhanced incorporation of a nutrient essential for cell growth.

Since drug resistance can develop on the basis of alterations in the intracellular accumulation of drug, it was essential for us to examine the uptake and release of 67Ga by R cells. Although the uptake of 67Ga correlates closely with the density of cellular Tf receptors (6), the increase in Tf receptors in the R cells did not lead to a corresponding increase in the uptake of 67Ga. Furthermore, the release of intracellular 67Ga from R and S cells was similar. Resistance to gallium nitrate therefore does not appear to result from decreased cellular accumulation or enhanced release of this metal from cells.

Although R cells displayed more Tf receptors than S cells, it should be appreciated that it is not the increase in Tf receptors per se which results in resistance to gallium. In previous studies utilizing a defined serum-free system, we have clearly shown that acute exposure of (sensitive) HL60 cells to Tf-Ga results in an inhibition of cellular iron incorporation and a subsequent increase in cellular Tf receptors over 24-48 h. However, despite the increase in Tf receptors, iron uptake by S cells is consistently inhibited by gallium at all stages of proliferation (7). The key difference between R and S cells is the finding that iron uptake by R cells is not continuously inhibited by gallium. R cells at confluency and replating do have an increased number of cell surface Tf receptors (in contrast to S cells which have low numbers of Tf receptors at replating). However, it is the increase in Tf receptors coupled with enhanced iron uptake which enables R cells to incorporate iron in sufficient amounts to support DNA synthesis and other critical iron-dependent processes.

Cellular requirements for iron during DNA synthesis appear to be related to the increased activity of the iron-containing M2 subunit of ribonucleotide reductase during the S phase of the cell cycle. The activity of this enzyme, as determined by ESR spectroscopy and measurements of cellular deoxyribonucleotide content, is decreased following exposure of HL60 cells to Tf-Ga (2). While the inhibitory effect of gallium on ribonucleotide reductase is believed to be secondary to a decrease in intracellular iron, it is possible that gallium may also have a more direct effect on this enzyme. Cells resistant to agents known to act specifically on the M2 subunit (e.g., hydroxyurea) display an overproduction of the M2 subunit and an increase in its ESR signal (13). In our studies, the ESR signals from S and R cells were found to be similar when examined at 24 h, under conditions of equal growth rate (i.e., in the absence of gallium). On further comparison of the ESR spectra from S and R cells, it is obvious that in the presence of increasing concentrations of gallium, the signal from S cells was diminished while signal from R cells was relatively unchanged, even at 960 μM of gallium nitrate. We interpret these results in the following way: In the presence of 84 μM gallium, iron uptake by S cells is inhibited and thus less intracellular iron is available to support the activity of the iron-dependent M2 subunit of ribonucleotide reductase. Accordingly, the ESR signal of S cells diminishes in the presence of gallium. In contrast, iron uptake by R cells is not inhibited by gallium; therefore sufficient intracellular iron is available to support the activity (ESR signal) of the M2 subunit. Since the ESR signals from S and R cells are similar under conditions of equal proliferation, it would appear that R cells are not resistant to gallium by virtue of increased activity of the M2 subunit of ribonucleotide reductase during the first 24 of subculture. Clearly, further investigations involving direct assay of ribonucleotide reductase are needed to assess the activity of this enzyme in R cells.

In addition to developing resistance to gallium through an
increase in Tf receptors and iron uptake, our studies also provide indirect evidence that R cells may utilize iron more efficiently than S cells. This conclusion is based upon a comparison of the ferritin content of the two cell populations. Ferritin synthesis is directly stimulated by iron and is increased or decreased when iron delivery to cells is increased or decreased (20, 21). Since iron uptake by R cells was greater than S cells, both cell populations would be expected to contain at least equivalent amounts of ferritin. However, the ferritin content of R cells was less than one-third that of S cells. This suggests that a significant portion of iron incorporated by R cells is not available to stimulate ferritin synthesis and is instead utilized more efficiently for cell growth.

Gallium has been shown to share several properties with iron with respect to Tf binding (4), cellular uptake by Tf receptors (5, 6), and incorporation into ferritin (6, 22). While Tf-Ga and Tf-Fe compete for initial entry into the cell via Tf receptor-mediated endocytosis, subsequent intracellular interactions between the two metals remain to be defined. Gallium and iron present in intracellular "pools" may compete for binding to macromolecules essential for cellular function. Since the incorporation of $^{67}$Ga into S and R cells was similar, it could be postulated that R cells overcome the cytotoxicity of gallium through enhanced incorporation of iron and expansion of the iron pool relative to the gallium pool. Maintenance of such a pool would involve shunting of iron through intracellular compartments whereby iron is unavailable to stimulate ferritin synthesis and is instead utilized more efficiently for cell growth.

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