Effects of Different Transferrin Forms on Transferrin Receptor Expression, Iron Uptake, and Cellular Proliferation of Human Leukemic HL60 Cells

Mechanisms Responsible for the Specific Cytotoxicity of Transferrin-Gallium

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Abstract

We have previously shown that human leukemic cells proliferate normally in serum-free media containing various transferrin forms, but the addition of transferrin-gallium leads to inhibition of cellular proliferation. Because gallium has therapeutic potential, the effects of transferrin-gallium on leukemic cell proliferation, transferrin receptor expression, and cellular iron utilization were studied. The cytotoxicity of gallium is considerably enhanced by its binding to transferrin and cytotoxicity can be reversed by transferrin-iron but not by other transferrin forms. Exposure to transferrin-gallium leads to a marked increase in cell surface transferrin binding sites, but despite this, cellular ^{59}Fe incorporation is inappropriately low. Although shunting of transferrin-gallium to another cellular compartment has not been ruled out, other studies suggest that transferrin-gallium impairs intracellular release of ^{59}Fe from transferrin by interfering with processes responsible for intracellular acidification. These studies, taken together, demonstrate that inhibition of cellular iron incorporation by transferrin-gallium is a prerequisite for inhibition of cellular proliferation.

Introduction

The receptor for transferrin (Tf),[1] the plasma Fe transport protein (1), was initially characterized on hemoglobin-producing cells and placental cells (2–4), but the more recent finding of increased Tf receptors on rapidly proliferating cells (5–9) has generated considerable interest. This latter finding underscores the importance of Tf for cellular proliferation (10). Although the most likely explanation for the function of transferrin in cellular proliferation is related to Fe transport, the exact nature of this requirement has not been defined, particularly in that Tf avidly binds a number of other metals, including zinc (12) and gallium (13, 14). Under physiologic conditions in vivo approximately one third of plasma Tf is Fe-saturated, leaving the remainder of the protein free to bind other metals. Thus, the ability of Tf to bind metals other than iron, may not only have previously undefined functions, but at present has medical diagnostic, therapeutic, and potentially toxic implications. Both in vitro and animal studies have demonstrated that tumor uptake of ^{67}Ga (an isotope commonly used for tumor detection in vivo) is enhanced by Tf (15–17), and it has been suggested that tumor uptake of ^{67}Ga is mediated by cell surface Tf receptor binding of Tf-^{67}Ga (18).

We have recently demonstrated that human promyelocytic leukemic (HL60) cells proliferate normally in serum-free media containing 25–150 μg/ml of Tf-Fe or apotransferrin (apoTf) as a necessary constituent. However, when no Tf or Tf is added as Tf-Ga, inhibition of proliferation is seen (19). Fluorocytometric analysis of Tf-Ga–treated cells indicated that there was an increase in immunofluorescent cell surface Tf receptor density and that with arrest of proliferation, cells accumulated in the S and G_2+M phases of the cell cycle (19). Furthermore, clinical studies have demonstrated the effectiveness of continuous intravenous infusion of gallium nitrate against lymphomas (20), tumors known to express Tf receptors in vivo (21). Additionally, some patients treated with gallium nitrate have developed microcytic hypochromic anemia suggesting that gallium may interfere with cellular Fe metabolism (20).

In order to better understand the mechanism of cytotoxicity of gallium and more specifically its effects on cellular Fe metabolism, we have studied the effect of Tf-Ga on leukemic cell Tf receptor expression, Fe uptake, ferritin synthesis, and intracellular release of Fe from Tf. We have compared the effects of Tf-Ga with apoTf, Tf-Fe, Tf-Cu, and Tf-Zn—Tf forms that are not cytotoxic in relatively low concentration. These studies may, in part, explain the mechanism of cytotoxicity of Tf-Ga and add support to the hypothesis that Fe is an important nutrient for maintenance of cellular proliferation.

Methods

Materials. Human apoTf, ferric chloride, zinc sulfate, copper sulfate, and fluorescein isothiocyanate (FITC) (isomer I on celite) were purchased from Sigma Chemical Co. (St. Louis, MO). Gallium nitrate was obtained from Alfa Products (Danvers, MA). Goat anti-rabbit IgG was obtained from Calbiochem-Behring Corp. (San Diego, CA). ^{125}I-sodium iodide and $^{59}FeCl_3$ were obtained from New England Nuclear (Boston, MA) and Amersham Corp. (Arlington Heights, IL), respectively. Saturation of apoTf with ^{59}Fe and iodination of ^{59}Fe-Tf were performed as previously described (8).

Preparation of various Tf forms. Tf-Fe, Tf-Ga, Tf-Zn, and Tf-Cu were prepared by using modifications of previously described methods (11, 13, 14, 22). For the respective preparations, 3 mol of metal (as ferric chloride, gallium nitrate, zinc sulfate, or copper sulfate) were added to each mole of apoTf dissolved in 20 mM acetic acid, 150 mM NaCl, pH 3.5. The pH of this solution was slowly raised in gradual increments to 7.4 with 1 M $NaHCO_3$ resulting in a final concentration of 30 mM $NaHCO_3$. Saturation of Tf by the different metals was confirmed spectrophotometrically using a DU-40 spectrophotometer (Beckman Instruments, Inc., Palo Alto, CA) by measuring the change in absorbance at

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1. *Abbreviations used in this paper:* apoTf, apotransferrin; EBSS, Earle's balanced salt solution; FITC, fluorescein isothiocyanate; Tf, transferrin.

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wavelengths of 242 nm (Ga, Zn), 530 nm (Cu), and 465 nm (Fe). For each metal Tf formed, saturation of both metal binding sites was confirmed by noting that the change in absorbance occurred progressively with the addition of increasing molar concentrations of metal until a 3 M excess of metal to Tf had been reached. Subsequently, for each Tf form the continued addition of metal to 6 M excess resulted in a <10% change in absorbance. Additionally, the change in absorbance noted at 242 nm agreed with previous data that confirmed binding of Zn (10) and Ga (13) to both Tf metal-binding sites, whereas the absorbance at 465 nm confirmed Fe saturation of Tf (22).

Stock solutions of Tf forms (10 mg protein/ml) were thus prepared and used for subsequent experiments. As Fe contamination of the various preparations would be expected, atomic absorption spectroscopy for Fe was performed by Coors Spectroscopy Laboratory (Denver, CO) using a Perkin-Elmer 5000 (Perkin-Elmer Corp., Norwalk, CT) with a flame source, and the Fe contamination of the various Tf stock solutions was 100–200 ng/ml (enough for 1% Tf saturation). More importantly, the media used in the cell culture experiments (RPMI 1640) was contaminated with 200 ng/ml of Fe (enough Fe to saturate about 150 μg of Tf).

Cells and cell growth studies. Human promyelocytic leukemia (HL60) cells were maintained in stock flasks in RPMI 1640 media containing 10% fetal calf serum with penicillin (200 U/ml) and streptomycin (0.2 mg/ml). Cells were grown in an atmosphere of 5% CO_2 at 37°C. Except for initial studies of cell growth in serum-supplemented media in response to different concentrations of gallium nitrate and Tf-Ga, all subsequent experiments were performed on cells grown in suspension in serum-free RPMI 1640 media supplemented with 5 μg/ml insulin (defined media) and varying concentrations of the different Tf forms as previously described (18). In studies examining the cytotoxicity of gallium nitrate and Tf-Ga in serum-supplemented media, cells were plated at 5×10^5/ml in media containing increasing concentrations of gallium nitrate or Tf-Ga and the effect of the two forms of gallium on cellular proliferation was noted.

For experiments of cell growth in defined media, HL60 cells were first grown to confluency and then plated at a density of 0.75×10^6/ml in defined media containing the Tf form under study.

^{125}I-Tf binding studies. Aliquots of cells growing in serum-free media containing apoTf, Tf-Fe, Tf-Ga, and Tf-Zn were harvested at 24, 48 and 72 h after initial plating. The cells were washed twice in ice-cold 10 mM KPO_4, 150 mM NaCl, pH 7.4, (phosphate-buffered saline) buffer containing 0.1% bovine serum albumin (PBS-BSA), and the cell pellets were diluted in 15 ml PBS-BSA and incubated at 37°C for 30 min. This 37°C "preincubation" step was performed to deplete the cells of any receptor-bound Tf and make available all of the Tf binding sites for the ^{125}I-Tf binding assay. Prior incubation of cells with ^{125}I-Tf, or immunoassays for Tf performed on cell lysates documented that >95% of bound Tf was removed with this preincubation step. Additionally, cells subjected to a second incubation step showed no difference in ^{125}I-Tf binding as compared with cells incubated once.

After the preincubation step, the cells were cooled to 4°C, washed once more with ice-cold PBS-BSA, and then resuspended in 2–3 ml of the same buffer. ^{125}I-Tf binding studies performed on these cells utilized a previously described assay (8, 18). Incubations were carried out at 4°C for 4 h, a time when equilibrium of binding was reached. Specific binding was determined as previously described (8) and maximal Tf binding was calculated according to the method of Scatchard (23).

^{59}Fe-Tf uptake studies. ^{59}Fe-Tf (2 μg Tf per ml) was added to cells subcultured in media containing 50 μg/ml of either apoTf, Tf-Fe, Tf-Ga, or Tf-Zn. At this Tf concentration, cells exposed to different Tf forms all showed the same rate of proliferation. After incubation for varying lengths of time aliquots of cells were harvested, and cell counts were performed. The cells were then washed and the cell pellet was counted for radioactivity.

Measurement of cellular ferritin content. Cells grown under different conditions were harvested, washed with PBS-BSA, and resuspended in 1 ml of PBS-BSA containing 0.1% Triton X-100. The cells were disrupted by sonication and incubated overnight at 4°C. Cellular debris was removed by centrifugation at 10,000 *g* in a J21-2M centrifuge (Beckman Instruments, Inc.) and the cytoplasmic supernatant was assayed for ferritin content using radiolabeled human spleen ferritin and a double-antibody radioimmunoassay (Amersham Corp.) as previously utilized for measuring cellular ferritin content (24).

Preparation of cellular lysates for Tf immunoassay. In order to study the effect of different Tf forms on the cellular uptake and intracellular release of ^{59}Fe, HL60 cells plated at 10^6/ml in media containing 50 μg/ml of either apoTf, Tf-Fe, Tf-Ga, or Tf-Zn were incubated at 37°C for 2 h when Tf-^{59}Fe (2 μg/ml) was added and the incubation continued for an additional 4 h. After the 6-h incubation, $5–10 \times 10^6$ cells were transferred to 15-ml conical tubes and rapidly cooled to 4°C and the supernatant was aspirated. The cells were washed three times with ice-cold PBS-BSA and resuspended in 1 ml of PBS-BSA buffer containing 0.1% Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5 IU/ml aprotinin. The cells were disrupted by sonication, incubated overnight at 4°C, and then centrifuged at 10,000 *g* in a J2-21M centrifuge (Beckman Instruments, Inc.) to remove cellular debris (which contained <10% of the total radioactivity in the sample). Aliquots from the cell extracts thus prepared were assayed for immunoreactive Tf content and used in immunoprecipitation experiments described below.

In separate experiments, pronase treatment (100 μg/ml for 1 h at 4°C) of aliquots of cells exposed to each different form of media documented that, consistently, 2–3% of the radioactivity could be released from each set of cells over and above that released by washing without pronase, indicating that this percentage of ^{59}Fe was on the cell surface or "trapped" between cells and released by pronase. These pronase-treated cells were not used for the subsequent experiments, however, because the percentage of radioactivity released was low, and washing of cells after pronase treatment to remove the enzyme resulted in obvious visible damage to the cells.

Tf radioimmunoassay. Immunoassay for Tf in cellular lysates was performed as previously described (4) with one major modification. In this modification, instead of previous assays using ammonium sulfate to precipitate the Tf antibody complex, polyethylene glycol (final concentration 4.5%) was added to each tube. The tubes were centrifuged at 20,000 *g* and the supernatants aspirated, and the pellets counted for radioactivity. A standard curve obtained using increasing amounts of nonradioactive Tf in the assay showed sensitivity ranging from 10 to 200 ng.

^{59}Fe-Tf immunoprecipitation. Aliquots of the cellular lysates were incubated with 10 μl of undiluted rabbit anti-Tf antibody for 1 h at 37°C in order to determine the amount of ^{59}Fe associated with Tf. Control experiments demonstrated that this amount of antisera was enough to precipitate more than 40 times the maximal amount of Tf present in the cell lysates. In these experiments, the incubation volume was made up to 400 μl with PBS-BSA containing 0.1% Triton X-100. After the addition of 100 μl of goat anti-rabbit IgG, the mixture was incubated for an additional 1 h at 37°C. After centrifugation at 20,000 *g*, the supernatants were carefully aspirated and the very small but visible precipitates and supernatants counted for ^{59}Fe radioactivity to determine the percentage of ^{59}Fe counts per minute associated with the immunoprecipitated Tf. Nonspecific precipitation of radioactivity obtained with control normal rabbit serum (3–4% of the total) was subtracted from the values obtained with specific rabbit anti-Tf antibody.

Cellular ^{125}I-Tf release experiments. Cells grown for various periods of time in serum-free media containing different Tf forms were harvested and cooled to 4°C, and nonradioactive cell-bound Tf was removed and the cells were washed as described above for ^{125}I-Tf binding experiments. The washed cells were then incubated with ^{125}I-Tf using the same cell number and Tf concentration as described above for binding assays, but this time the incubation was performed for 30 min at 37°C. After the 37°C incubation, the cells were cooled to 4°C, washed with buffer, and then resuspended in 1 ml of 37°C serum-free media with or without 50 μg of the Tf form the cells were originally grown in. Aliquots of the cell suspension were removed from each tube after 0, 5, 10, 15, 30, and 60 min of incubation and each 50-μl aliquot was transferred to a tube con-

taining 1 ml of ice-cold buffer. The tubes were immediately centrifuged and the separated cell pellets and supernatants were counted for radioactivity to determine the percentage of cell-associated ^{125}I-Tf.

^{59}Fe-Tf release experiments. The effects of two non-Fe Tf metal preparations (Tf-Ga and Tf-Zn) on cellular ^{59}Fe-Tf uptake and release were systemically studied. Cells plated in media containing 50 μg/ml of either Tf-Ga or Tf-Zn were harvested after a 24-h incubation and washed twice with PBS-BSA. Cells in each sample (10^7) were resuspended in 1 ml of buffer containing 12 ng of Fe as ^{59}Fe-Tf. After incubation at 4°C for 4 h, the cell suspension was washed free of unbound ^{59}Fe-Tf and the cell pellet was resuspended in 200 μl of buffer and incubated at 37°C for 1 min. 1 ml of media at 37°C containing 50 μg/ml of Tf-Ga or Tf-Zn was added to the corresponding pellets. Small aliquots (50 μl) of cell suspension were removed after 0, 2, 6, 10, 30, and 60 min of this incubation and rapidly cooled by transferring the aliquots to tubes containing 1 ml of ice-cold buffer. The tubes were promptly centrifuged and the separated pellets and supernatants were counted to determine the percentage of pellet-associated ^{59}Fe.

Preparation of FITC-labeled Tf. Tf was labeled with FITC using a modification of a previously reported method (25). Tf-Fe and Tf-Ga (10 mg/ml) were first prepared as described above and then dialyzed against 0.1 M sodium borate, pH 9.0. No change in the saturation of Tf by either metal was noted after dialysis. FITC I (2.5 mg of 10% wt/wt on celite) was added to each milliliter of Tf metal with continuous mixing at 4°C for 30 min. FITC-celite particles were removed by high-speed centrifugation and the supernatant was dialyzed for 24 h against several changes of 150 mM NaCl 10 mM Tris, pH 7.4, to remove unbound FITC. Dialysis was continued until the dialysis buffer was clear. The FITC-Tf thus prepared was incubated in varying dilutions with HL60 cells at 4 and 37°C for 30–60 min to enable binding of FITC-Tf to cellular Tf receptors. After three washes with Earle's balanced salt solution (EBSS) to remove unbound FITC-Tf, the cells were examined directly by fluorescent microscopy to ensure that FITC-Tf had indeed bound to the cells and was internalized at 37°C. Cell fluorescence could be completely blocked by prior addition of a 100-fold excess of nonfluorescent Tf, confirming that both preparations of FITC-Tf bound specifically to cellular Tf receptors.

Spectrofluorometer studies. Spectrofluorometric analysis of cells was performed using an Aminco SPF-500 ratio spectrofluorometer (American Instrument Co., Silver Spring, MD) (25, 26). In all samples the fluorescence intensities at 520 nm were read using separate excitation wavelengths of 490 and 460 nm, respectively. A calibration curve was obtained by adjusting the pH of the FITC-Tf standard sample through a pH range of 7.5–5.0 and noting corresponding changes in fluorescence intensity. HL60 cells (10^7), grown in RPMI 1640 and 10% fetal calf serum (FCS), were washed with EBSS and incubated at 4°C for 2 h in 1 ml of the same buffer containing 200–500 μg/ml FITC-Tf-Fe or FITC-Tf-Ga. Cells were then washed twice with 50 ml of ice-cold EBSS and resuspended in 1 ml of buffer. Prior to spectrofluorometric analysis, the cells were examined directly by fluorescent microscopy to confirm binding of the FITC-Tf to cell surface Tf receptors. The cells were then transferred to cuvettes and fluorescence intensities at 520 nm read separately for excitation wavelengths of 490 and 460 nm. A circulating water bath was used to maintain a constant cuvette temperature at 4°C. The cells were then removed from the cuvettes, incubated at 37°C for 5 min to allow internalization of cell surface receptor-bound FITC-Tf, and then after being cooled again to 4°C, their fluorescence intensities were determined as before. Blank (background) readings obtained on identical unlabeled Tf-treated cells were subtracted from the fluorescence intensity readings of the FITC-Tf–treated cells.

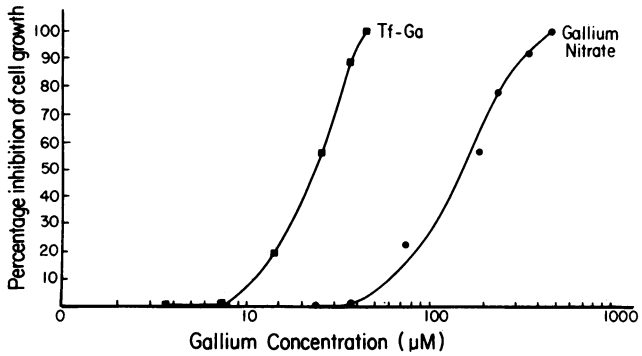

Figure 1. Effect of gallium on proliferation of HL60 cells. HL60 cells were plated at a density of 5×10^5/ml in complete media (RPMI 1640 with 10% FCS). Gallium as gallium nitrate or transferrin gallium (Tf-Ga) was added to different flasks and cell counts were performed after 72 h of culture.

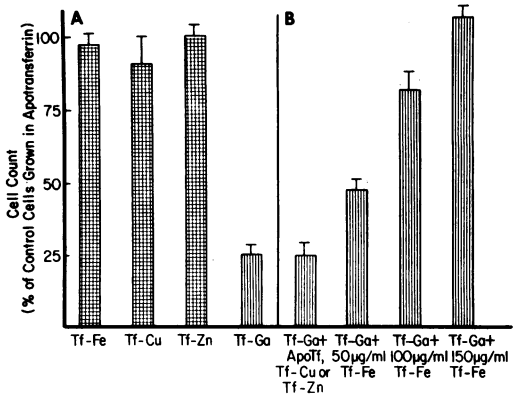

Figure 2. HL60 growth in the presence of different Tf forms. (*A*) Cells were plated in serum-free media containing 150 μg/ml of different Tf forms and cell growth was compared with cells grown in the media containing 150 μg/ml of apoTf. (*B*) 150 μg/ml of either apoTf, Tf-Cu, Tf-Zn, or Tf-Fe in concentrations of 50, 100, and 150 μg/ml were added to the media containing 150 μg/ml of Tf-Ga. All cell counts (±range) were performed after 72 h of incubation.

Results

Enhancement of Ga cytotoxicity by binding of Ga to Tf. HL60 cells grown in RPMI 1640 media supplemented with 10% FCS showed a progressive decrease in proliferation with addition of increasing doses of Ga added as $GaNO_3$. When Ga was added as Tf-Ga, inhibition of growth was also dose-related and the effect of Ga was enhanced about 10-fold when it was first bound to Tf (Fig. 1). This 10-fold difference is a conservative estimate in that the bovine Tf present in this serum-supplemented media would presumably have the ability to bind and transport gallium when added as gallium nitrate. In subsequent experiments using serum-free media (defined media), even lower doses of Tf-Ga were required to inhibit cell growth. In the same serum-free system, cells grown in media containing similar concentrations of apoTf, Tf-Fe, Tf-Cu, or Tf-Zn grew normally (Fig. 2 *A*).

Reversal of the Tf-Ga inhibition of cellular proliferation. The simultaneous addition of Tf-Fe to media containing cytotoxic concentrations of Tf-Ga resulted in dose-related restoration of cellular proliferation (Fig. 2 *A* and *B*). The reversal of Tf-Ga inhibition of cellular proliferation by equimolar concentrations of Tf-Fe was greatest when the two forms of Tf metal were added to the media simultaneously at the time of the initial plating. When the Tf-Fe was added 24 or 48 h after the addition of Tf-

Ga to the media, cells resumed proliferation but a lag was noted in that the cells only attained 50–75% of the control cell number by 72 h, but by 96 h the cell counts were the same as control. The addition of apoTf, Tf-Zn, or Tf-Cu in equimolar concentrations showed no measurable reversal of the Tf-Ga inhibition of cell growth.

When the concentration of apoTf or Tf-Zn in the media were increased to 2 mg/ml, marked inhibition of proliferation and eventual cell death were observed. Cells exposed to >200 μg/ml of Tf-Cu proliferated at a slower rate with evidence for a small, but significant percentage of cells becoming fragmented and nonviable; with a small increase in Tf-Cu concentration to 300 μg/ml most of the cells rapidly lost viability and fragmented, as indicative of Cu toxicity. This effect of Tf-Cu is far different from the effects seen with Tf-Ga in which cells show a dose-related effect on inhibition of proliferation but still maintained viability for at least 1 d after total inhibition of proliferation resulted. Tf-Cu added to media at lower concentrations showed evidence for both increased Tf receptor expression and increased ability to incorporate radioactive Fe from media. However, Tf-Cu was not used systematically because its use was limited by the narrow range of concentrations that could be used before toxicity occurred.

Our previous studies had indicated that Tf receptor density was increased on the surface of Tf-Ga–treated cells, as evidenced by an increase in immunoreactive surface Tf receptor (19). Initial ^{125}I-Tf binding studies performed on cells 24 h after incubation with 150 μg/ml Tf-Ga documented that there was a marked increase in Tf binding sites on cells exposed to Tf-Ga as compared with other Tf forms, and these cells exhibited a marked decrease in ability to incorporate ^{59}Fe when a trace amount of ^{59}Fe-Tf was added to the media. Further evidence for decreased Fe uptake by cells treated with 150 μg/ml of Tf-Ga was provided by markedly decreased cellular ferritin content. However, although these cells treated with 150 μg/ml of Tf-Ga were >95% viable at 24 and 48 h after treatment, the following studies performed systematically on Tf-Ga–treated cells utilized 50 μg/ml of Tf-Ga, a concentration that allowed cells to proliferate normally for at least 72 h.

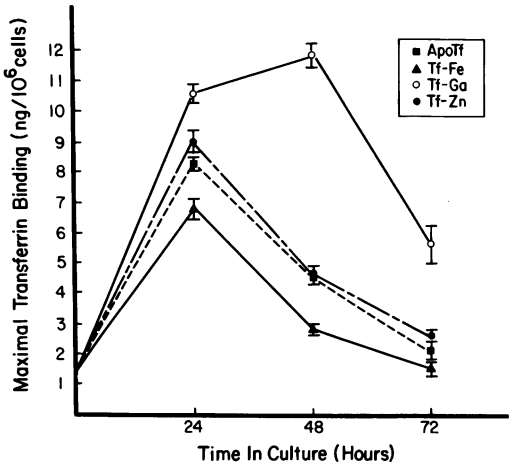

Figure 3. Measurement of cellular ^{125}I-Tf binding sites after cell growth in media containing different transferrin-metals. HL60 cells were plated at 1 × 10^6/ml in serum-free media containing 50 μg/ml of different transferrin-metals. After 24, 48, and 72 h, cells were harvested, receptor-bound transferrin was eluted, and assays for transferrin binding were performed. Maximal transferrin binding was determined. All points represent the mean of four experiments±SD.

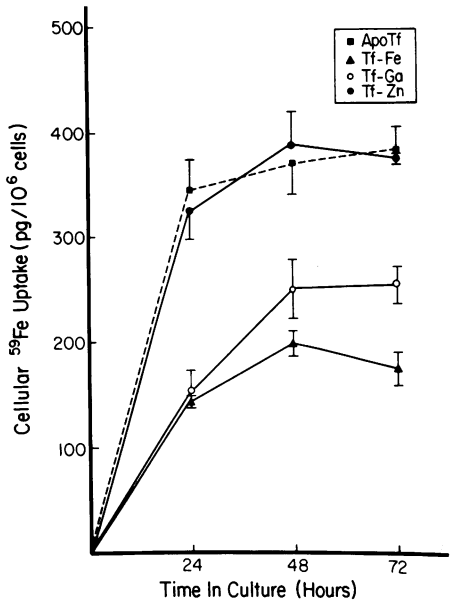

Figure 4. ^{59}Fe uptake by HL60 cells grown in the presence of different transferrin-forms. Cells were plated in media containing 50 μg/ml of the different transferrin-forms and ^{59}Fe-Tf (2 μg/ml) was added to each flask. After 24, 48, and 72 h of incubation cells were harvested and washed and cell pellets were counted for ^{59}Fe radioactivity. All points represent the mean of three experiments±SD.

Measurement of cellular ^{125}I-Tf binding after growth in media containing different Tf forms. Cells plated in defined media containing 50 μg/ml of different Tf forms were harvested, washed by centrifugation, and preincubated at 37°C to remove bound nonradioactive Tf as described in Methods. As shown in Fig. 3, at 24 h after subculture, cells treated with Tf-Fe had decreased Tf-binding sites as compared with cells treated with apoTf or Tf-Zn. This difference was also present at 48 h and confirms the results of previous studies which have indicated that cells that incorporate more Fe exhibit a subsequent decrease in expression of Tf receptor (26). Cells treated with Tf-Ga exhibit a marked increase in Tf binding sites at all time points as compared with other cells (Fig. 3). Binding studies performed at 37°C demonstrated similar results to those shown in Fig. 3, except there were about twice the number of binding sites measured for all cell aliquots as compared with 4°C, a finding we have previously described with HL60 cells (18).

The differences in ^{125}I-Tf binding to cells grown in media containing the different Tf forms reflect actual differences in the number of binding sites because cells treated in all the Tf forms had the same affinity for ^{125}I-Tf (0.8–1.0 × 10^{-9} M). In other studies, inhibition of ^{125}I-Tf binding (5 ng/Tf) to cells grown in apoTf was the same using a wide range of concentrations (1 ng to 1 μg) of either Tf-Fe, Tf-Ga, Tf-Zn, indicating that all three Tf forms had similiar affinities for the Tf receptor.

^{59}Fe uptake by cells grown in defined media containing different Tf forms. ^{59}Fe-Tf (see Methods) was added to cells in media containing 50 μg/ml of the different Tf forms. Because previous

studies have indicated that radioactive Fe incorporation by cells is directly related to the surface density of Tf receptors (8, 26), it is not surprising that cells treated with apoTf or Tf-Zn show an increase in radioactive Fe incorporation as compared with cells treated with Tf-Fe (Fig. 4). However, cells treated with Tf-Ga show decreased incorporation of ^{59}Fe relative to cells treated with apoTf and Tf-Zn in spite of a high density of surface ^{125}I-Tf binding sites. This discrepancy between increased surface Tf receptors and low Fe uptake is most striking at the 48-h time point when, despite the 2.5-fold increase in Tf binding as compared with apoTf and Tf-Zn–treated cells, Tf-Ga cells incorporate 35% less ^{59}Fe (compare Figs. 3 and 4).

In other experiments ^{59}Fe-Tf was added after cells had grown for 24 or 48 h in media containing the different Tf form, and ^{59}Fe uptake by cells over the next 6–24 h determined. Again, the uptake of ^{59}Fe was persistently low in cells grown in Tf-Ga as compared with other Tf forms. This effect was seen as early as 6 h after incubation with Tf-Ga, a time period when there was no difference in Tf receptor density on the various cells demonstrating that the decreased uptake of ^{59}Fe by Tf-Ga–treated cells preceded any change in Tf receptor number. In further experiments, we also determined that this Tf-Ga effect on decreased ^{59}Fe uptake was reversible within at least 6 h.

We investigated the possibility that differences in ^{59}Fe uptake might be due to differences in ^{59}Fe-Tf binding to cells in media containing the various Tf metals. Therefore, HL60 cells originally grown in apoTf were aliquotted in media at 4°C containing 50 μg/ml of the different Tf forms. ^{59}Fe-Tf was added to the media and the incubation was continued for 6 h at 4°C. The cells were then harvested, pelleted, and counted for ^{59}Fe radioactivity. In duplicate experiments obtained for cells incubated with each Tf form, there was <5% variance and cells plated in all the different media bound the same amount of ^{59}Fe at 4°C (<10% variation). Thus, the decrease in ^{59}Fe incorporation seen with cells grown in the presence of Tf-Ga is not the result of decreased surface binding of ^{59}Fe-Tf, but the results suggest that the differences are due to some event which occurs after ^{59}Fe-Tf is bound to the cell surface.

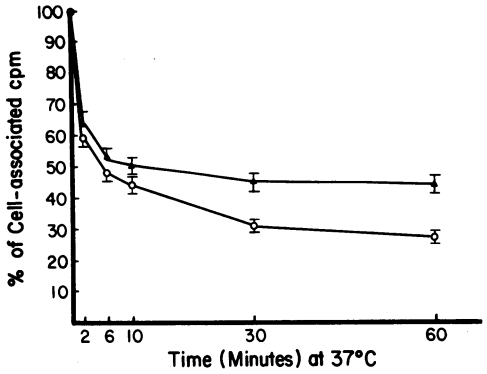

Figure 6. ^{59}Fe-Tf release from cells. HL60 cells grown for 24 h in media containing 50 μg/ml of Tf-Ga (○) of Tf-Zn (▲) were harvested, washed, and incubated with ^{59}Fe-Tf for 4 h at 4°C to permit binding of ^{59}Fe-Tf to cell surface transferrin receptors. Unbound ^{59}Fe-Tf was removed by washing and the cells were resuspended in 200 μl of buffer and incubated at 37°C for 1 min, and 1 ml of warm media containing 50 μg/ml of Tf-Ga or Tf-Zn was then added to the corresponding cells and the incubation was continued at 37°C. Aliquots of cell suspensions were removed at 0, 2, 6, 10, 30, and 60 min after addition of warm media, rapidly cooled to 4°C, and centrifuged. Cell pellets and supernatants were then counted separately for ^{59}Fe radioactivity and the percentage of radioactivity remaining in the cells was determined. Points represent mean of three experiments±SD.

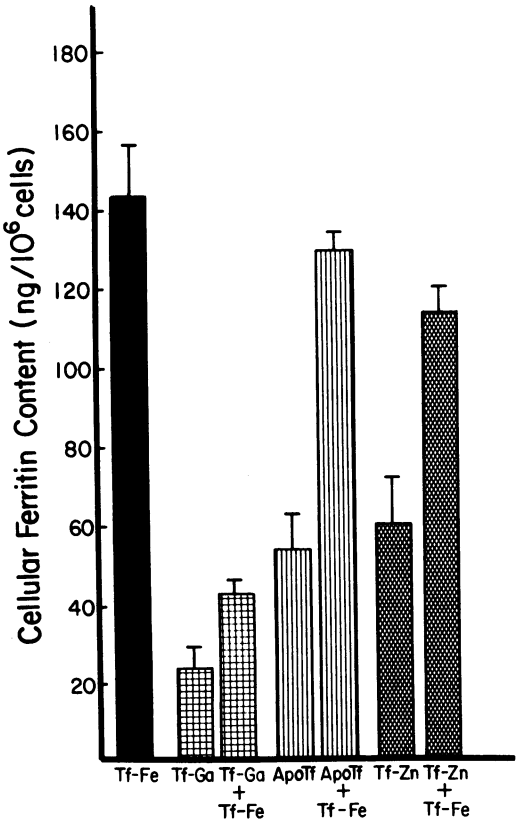

Figure 5. Cellular ferritin content after cell growth in media containing different transferrin-metals. Cells were grown in media containing 50 μg/ml of apoTf, Tf-Ga, or Tf-Zn with or without the addition of 50 μg/ml of Tf-Fe. Cellular ferritin content was measured by radioimmunoassay after 72 h of incubation. Bars represent mean of three experiments±SD.

Cellular ferritin content. Measurements of ferritin content of cells grown for 72 h in media containing 50 μg/ml of Tf-Fe, apoTf, Tf-Ga, or Tf-Zn were performed. Cells grown in Tf-Fe medium alone contained the highest amount of ferritin while the cells grown in Tf-Ga medium contained a very low ferritin content, and cells grown in apoTf or Tf-Zn media contained intermediate amounts (Fig. 5). With the addition of Tf-Fe to their respective defined media, the apoTf and Tf-Zn cells increased their ferritin content to levels found in cells grown in Tf-Fe media alone; Tf-Ga–treated cells, however, still exhibited a profound decrease in ferritin content, which based on previous studies (27) is highly indicative of the fact that Tf-Ga–treated cells have decreased Fe incorporation over this period of exposure.

^{125}I-Tf release from cells grown in the presence of different Tf forms. We then studied the kinetics of release of ^{125}I-Tf previously internalized by HL60 cells grown in various Tf forms. Cells exposed to four Tf forms (apoTf, Tf-Fe, Tf-Ga, and Tf-Zn) displayed almost identical ^{125}I-Tf release kinetics. The pres-

Table I. Measurements of Total Cellular Immunoreactive Transferrin and Incorporated ^{59}Fe Bound to Transferrin in Cells Grown in Different Media

Transferrin form present in media	Cellular immunoreactive transferrin	Total ^{59}Fe incorporated/10^6 cells	% ^{59}Fe immunoprecipitated by anti-transferrin antiserum	^{59}Fe-Tf incorporated
	ng/10^6 cells	*cpm*	*%*	*ng/10^6 cells*
Apo-Tf	76±17	1,002±239	9.6±0.7	2.98±0.9
Tf-Fe	92±18	668±52	9.8±1.3	2.00±0.5
Tf-Ga	87±18	483±34	21.2±2.3	3.15±0.7
Tf-Zn	89±21	886±196	11.0±1.4	3.01±1.2

ence of 50 μg/ml of the corresponding Tf form in the "release media," as expected, enhanced ^{125}I-Tf release resulting in release of 85% of ^{125}I-Tf from the cell pellets after 1 h. In the absence of the corresponding Tf form in the release media, cell pellets had released only 60% of ^{125}I-Tf after 1 h. These latter studies were almost identical for cell aliquots previously exposed to different Tf forms, although in these experiments it might be hypothesized that any potential differences found in Tf-Ga–treated cells may have been reversed in that Tf-Ga was not present during the "release" period.

^{59}Fe release from HL60 cells. Because no differences in cellular ^{125}I-Tf release were noted, we measured release of ^{59}Fe once cells had bound ^{59}Fe-Tf. After exposure to either Tf-Ga or Tf-Zn, cells were permitted to first bind ^{59}Fe-Tf at the cell surface at 4°C, incorporate the ^{59}Fe at 37°C (for 1 min), and then release of ^{59}Fe into media containing Tf-Ga or Tf-Zn was measured. Binding of the ^{59}Fe-Tf at 4°C was the same for both sets of cells:

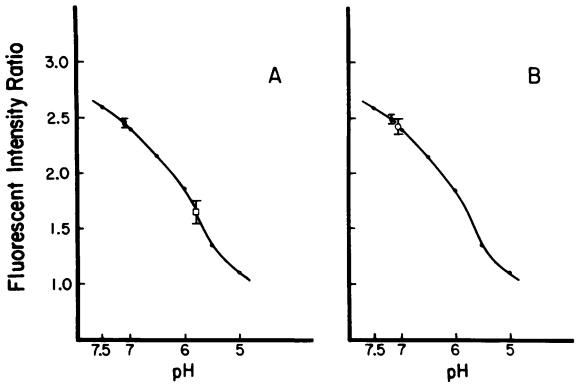

Figure 7. Changes in FITC-Tf fluorescence before and after internalization of FITC-Tf by HL60 cells. The fluorescence intensity ratio represents the ratio of fluorescence intensity at excitation wavelengths 490 nm/460 nm. The calibration curve was obtained by plotting changes in the fluorescence intensity ratio using a solution of each FITC-Tf-form at differing pH concentrations. HL60 cells were incubated at 4°C with either FITC-Tf-Fe or FITC-Tf-Ga to permit binding to cell surface transferrin receptors. After washing to remove unbound FITC-Tf the fluorescence intensities of each sample were measured at 4°C in a spectrofluorometer. The samples were then removed, incubated at 37°C to allow internalization of FITC-Tf, and then cooled again to 4°C and fluorescence intensities measured again. (*A*) Fluorescence intensity ratio with FITC-Tf-Fe at 4°C (■) and after the 37°C incubation (□). (*B*) Fluorescence intensity ratio with FITC-Tf-Ga at 4°C (●) and after the 37°C incubation (○). Each temperature determination represents mean±SD of three experiments.

starting counts per minute per 1×10^7 cells was 10,325±812 for Tf-Zn–treated cells and 10,680±935 for Tf-Ga–treated cells. Cellular ^{59}Fe release measured during the first 6 min for both Tf-Ga– and Tf-Zn–treated cells showed very similar ^{59}Fe release kinetics (Fig. 6). After 10 min of incubation at 37°C, 53% of the initial cell-associated ^{59}Fe is still associated with the Tf-Zn cells. This decreases to 45% at 30 min and remains relatively constant at 60 minutes. These kinetics are similar for identical experiments performed on cells exposed to Tf-Fe (although only about 3,900 cpm are initially bound per 1×10^7 cells, presumably owing to decreased Tf receptor expression): 42% of the radioactivity is associated with the cells at 30 min and 41% at 60 min. In contrast, the Tf-Ga–treated cells retain 44% of the initial cell-associated ^{59}Fe at 10 min, 31% after 30 min, and 27% after 60 min of incubation. Thus, after 60 min of incubation in their respective "release media" Tf-Ga–treated cells release 18% more ^{59}Fe than cells exposed to Tf-Zn even though both sets of cells bound a similar amount of ^{59}Fe-Tf at the beginning of the experiment (Fig. 6). Gel filtration experiments substantiated that >95% of the ^{59}Fe released from both aliquots of cells was bound to Tf. The initial rapid and equal release of ^{59}Fe from both sets of cells during the first 6 min may represent dissociation of ^{59}Fe-Tf from the cell surface both during the initial 1-min 37°C incubation and as a result of displacement of bound ^{59}Fe-Tf by Tf-Ga or Tf-Zn in the release media. On the other hand, subsequent cellular release of ^{59}Fe to the media (during 6–60 min) may represent release of incorporated ^{59}Fe. These experiments suggest, therefore, that the decrease in overall uptake of ^{59}Fe over time by Tf-Ga–treated cells may be due to accelerated release of ^{59}Fe after internalization.

Tf immunoprecipitation experiments. Assays for cellular Tf content and immunoprecipitation of total intracellular Tf were performed on solubilized cytoplasmic extracts of cells which had been plated in different Tf-containing media and incubated with ^{59}Fe-Tf. A 2-h incubation at 37°C followed by an additional 4 h with Tf-^{59}Fe were chosen because (*a*) at this time point (6 h) no significant difference in Tf receptor density is seen among cells exposed to the different Tf forms, and (*b*) enough ^{59}Fe is incorporated so that accurate measurements can be performed. As shown in Table I, ^{59}Fe incorporated is significantly less for Tf-Ga–treated cells ($P < 0.01$–0.02) than for cells incubated with other Tf forms. The intracellular Tf content, as determined by radioimmunoassay, was found to be similar in the cells exposed to the different Tf forms (Table I). In order to determine the amount of ^{59}Fe still associated with Tf after cellular uptake of ^{59}Fe-Tf, aliquots from the different cytoplasmic extracts were immunoprecipitated using undiluted anti-Tf antiserum. As

shown in Table I, 21.2% of the total ^{59}Fe counts per minute in the Tf-Ga–treated cells was associated with immunoprecipitable Tf. In contrast only 9.6%, 9.8%, and 11.0% of total intracellular ^{59}Fe counts per minute was associated with Tf in cells exposed to apoTf, Tf-Fe, and Tf-Zn, respectively.

As shown in the last column in Table I, based on the counts per minute of ^{59}Fe immunoprecipitated by anti-Tf antisera, the calculated amount of radioactive Tf in Tf-Ga cells, although not statistically different, is higher than the immunoprecipitable Tf found in cells treated with other Tf forms. Thus, the higher percentage of ^{59}Fe immunoprecipitated with anti-Tf antibody in Tf-Ga–treated cells, does not simply reflect the reduced deposition of iron per se during the 4-h loading period, but rather, these results indicate that an appropriate, if not higher, amount of ^{59}Fe-Tf is found Tf-Ga–treated cells. Thus, these data suggest that decreased iron uptake in the Tf-Ga–treated cells is due to impaired iron release from already incorporated ^{59}Fe-Tf.

Spectrofluorometric studies. Cells incorporate Fe by means of receptor-mediated endocytosis of Tf-Fe with the intracellular release of Fe from Tf occurring within an acidic nonlysosomal intracellular compartment (25, 28, 29). We sought to determine whether Tf-Ga interfered with cellular Fe incorporation by affecting the pH of this compartment.

The standard curve obtained for each FITC-Tf form (Fig. 7 *A* and *B*) shows that, as has been reported by others (25, 30), the 490-nm/460-nm ratio decreases with decreasing pH. In order to study pH changes within the cellular compartment after uptake of FITC-Tf-metal, untreated cells were first analyzed at 4°C after binding of FITC-Tf-Fe or FITC-Tf-Ga to their cell surfaces. Fluorescence intensity 490-nm/460-nm ratio readings were similar for both FITC-Tf-Fe and FITC-Tf-Ga confirming that at 4°C the FITC-Tf was present only on the cell surface and was at neutral pH (Fig. 7 *A* and *B*). After incubation at 37°C to allow internalization of surface receptor-bound FITC-Tf, the FITC-Tf-Fe cells displayed a drop in their fluorescence intensity ratio compatible with movement of the FITC-Tf-Fe into an acidic compartment (Fig. 7 *A*). The FITC-Tf-Ga cells, however, showed a markedly blunted decrease in fluorescence intensity after incubation at 37°C (Fig. 7 *B*). Because it was possible that the absence of fluorescence quenching in the FITC-Tf-Ga-treated cells could also result from impaired internalization of the surface-bound FITC-Tf-Ga, cells from both samples before and after the 37°C warm-up were examined by direct fluorescent microscopy. Cells at 4°C showed a distinct rim fluorescence with both FITC-Tf forms. After the 37°C incubation, a decrease in fluorescence and the appearance of intracellular fluorescence was noted to be (under "blinded" observation) qualitatively identical for both the FITC-Tf-Fe– and FITC-Tf-Ga–treated cells. Although these qualitative similarities suggest that the FITC-Tf-Ga is in the same intracellular compartment as Tf-Fe, it is certainly possible that the Tf-Ga complex is delivered to a different intracellular compartment than Tf-Fe. Alternatively, the absence of fluorescence quenching after cellular uptake of FITC-Tf-Ga may result from failure of these cells to attain an acidic pH within the same intracellular compartment and therefore result in decreased release of Fe from Tf.

Discussion

Measurements of Tf receptor using both Tf binding studies and immunoassay techniques indicate that events preceding cell division provide a regulatory stimulus for the synthesis and subsequent appearance of the Tf receptor on the cell surface (7, 8, 19, 26, 31, 32). These findings support other studies that have demonstrated that Tf is a necessary requirement for cellular proliferation (10, 33). Whether this Tf requirement is related to Fe transport has not been clarified. In previous studies, we demonstrated that human leukemic cells grown in a serum-free system showed normal proliferation in the presence of apoTf or Tf-Fe, but after exposure to Tf-Ga, proliferation ceased (19). Because radioactive Ga has been used in the past as a diagnostic agent for localization of tumors (19), and more recent studies have used Ga as a potential chemotherapeutic agent (20), we performed a number of experiments to define the biochemical effects associated with Ga treatment in vitro. These experiments are particularly important because the use of Ga as a chemotherapeutic agent has a great deal of potential, in that Ga therapy does not cause some of the more severe toxic effects associated with most chemotherapeutic agents (20).

Prior studies have suggested that the cellular uptake of radioactive gallium is enhanced by the addition of Tf to culture media in vitro (15–17), and the effect of gallium salt inhibition of cellular proliferation is also enhanced by the addition of Tf (34). In this manuscript, we have demonstrated that for HL60 cells Tf-Ga is a much more effective cytotoxic agent than gallium nitrate alone, and that proliferation is arrested with Tf-Ga in a dose-dependent fashion. Mixing studies demonstrate that Tf-Ga effects on cellular proliferation can be overcome by the presence of Tf-Fe, but not by other Tf forms. Thus, only Tf-Fe appears to be able to supply sufficient Fe to overcome the effects of Tf-Ga.

Further studies using lower concentrations of Tf-Ga, which allow cells to proliferate normally, better define the specific action of Tf-Ga on cellular Fe uptake. Thus, as compared with cells exposed to other Tf forms, cells grown in 50 μg/ml of Tf-Ga exhibit a marked relative decrease in ^{59}Fe incorporation demonstrating that impaired cellular Fe incorporation precedes the arrest of proliferation seen with higher doses of Tf-Ga. Additionally, this relative decrease in ^{59}Fe incorporation by Tf-Ga–treated cells could be seen as early as 6 h after initial plating, well before significant differences in Tf receptor expression were noted. In other experiments, Tf-Ga–treated cells exhibit a decrease in total Fe content as indirectly measured by decreased ferritin content as compared with cells exposed to other Tf forms.

The up-regulation of cell surface Tf receptors on cells exposed to Tf-Ga, apoTf, and Tf-Zn is almost certainly the result of decreased Fe uptake by these cells since other investigations using various Fe chelators have demonstrated up-regulation of Tf receptor synthesis, and expression, due to either decreased cellular Fe incorporation or chelation of an intracellular Fe pool (35–37). Thus, as shown in this report, depletion of intracellular Fe content results within 24 h in increased cell surface expression of the Tf receptor protein. However, although cells exposed to apoTf and Tf-Zn show the expected increase in incorporation of trace Fe, a result of increased Tf receptor expression, Tf-Ga–treated cells show a marked inappropriate decrease in Fe incorporation in spite of high Tf receptor expression.

Impaired intracellular Fe incorporation, therefore, is a specific effect of Ga since cells treated with Tf-Ga show an inability to release Fe from trace amounts of Tf-Fe after Tf is bound to the cell surface. Evidence for this defect is supported by the finding of a much higher percentage of intracellular radioactivity

identified as ^{59}Fe-Tf in Tf-Ga–treated cells as compared with cells exposed to other Tf forms. Additionally, although "shunting" of Tf-Ga to a different intracellular compartment has not been ruled out, experiments utilizing FITC-labeled Tf-forms suggest that cells treated with Tf-Ga have lost the ability to acidify the intracellular compartment that allows Fe to be released from Tf, and also allows for recycling of apoTf back to the cell surface. This effect may be specifically caused by free intracellular Ga, since even with a slight drop in pH, gallium might be released from Tf in this intracellular compartment. The effects of Ga appear to be more specific than other agents, including NH_4Cl (38), which neutralize intracellular acidic compartments but presumably have a wide variety of cellular effects. How Tf-Ga, or more specifically, Ga, interferes with acidification in the endosome or other processes yet to be defined, associated with Fe transport, has not been determined. Further understanding of gallium effects would appear to be important in understanding its beneficial therapeutic/toxic ratio in that Tf delivery of this metal is not associated with a multitude of toxicities seen with other forms of metal loading, including Cu, Pb, or Al (39–41). Toxicity from some of these metals may be due to variable inhibition of heme synthesis (39–41). In preliminary experiments, we have found that human reticulocytes incubated with Tf-Ga and a trace dose of ^{59}Fe-Tf show the appropriate decrease in cellular Fe incorporation, as with leukemic cells. However, Tf-Ga does not inhibit Fe incorporation into heme once Fe is internalized, indicating that the major effect of Tf-Ga on these cells is also related to inhibition of Fe incorporation from Tf-Fe.

The presence of Tf receptors on some rapidly proliferating tumors in vivo provides an opportunity to use Tf-Ga as a cytotoxic agent against these tumors. The National Cancer Institute presently lists eight open protocols utilizing gallium nitrate for the treatment of tumors of the prostate, kidney, testes, and small-cell lung cancer. Almost all of these regimens employ an intermittent rapid infusion treatment schedule that, based on the present studies, would appear to not be as effective as continuous infusion in allowing for sustained Tf saturation with the metal. The study using a 7-d regimen of continuous infusion of Ga, which was highly effective in the treatment of lymphomas, suggested that protein binding was at least partly responsible for sustained levels of the drug (20). Additionally, tumors with a relatively slow rate of proliferations such as prostatic cancer might not be expected to respond to Ga treatment. Therefore, further understanding of the interaction of Ga with Fe, Tf, and Tf receptors will lead to a more judicious and effective use of this agent in the treatment of specific malignancies.

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