Modulation of In Vitro and In Vivo T-Cell Responses by Transferrin-Gallium and Gallium Nitrate

By William R. Drobyski, Riaz U-Haq, David Majewski, and Christopher R. Chitambar

Gallium is a group IIIa metal that has efficacy in the therapy of malignant disorders such as lymphoma and urothelial tract tumors. Preclinical studies also indicate a role for gallium in autoimmune disorders, suggesting that gallium is able to modulate T-cell immune reactivity. The purpose of this study was to examine the in vitro and in vivo immunomodulatory action of gallium on T-cell function. Since gallium binds to transferrin in vitro, preclinical studies evaluated the effect of transferrin-gallium (Tf-Ga) on human T cells. Tf-Ga inhibited the mitogen-induced proliferative response of peripheral blood mononuclear cells (PBMC) in a dose-dependent fashion. Alloantigen-induced proliferation was also potently suppressed when evaluated in a mixed lymphocyte culture assay. Tf-Ga affected a significant reduction in the density of IL-2 receptors on activated T cells and a slight reduction in the number of CD3+CD25+ T cells in PHA-stimulated cultures. Neither secretion of interleukin-2 (IL-2) nor the induction of IL-2–stimulated lymphokine-activated killer activity, however, was inhibited by Tf-Ga. Tf-Ga produced significant upregulation of the transferrin receptor (CD71) in T cells as determined by flow cytometric analysis and northern blot assay, but did not affect the percentage of CD3+CD71+ T cells after mitogen stimulation. To assess the in vivo effects of gallium on alloreactive T cells, we evaluated the immunosuppressive effect of gallium in a murine model of graft-versus-host disease (GVHD). Administration of gallium significantly prolonged survival in mice undergoing severe GVHD, suggesting that gallium can ameliorate GVH reactivity. Collectively, these data demonstrate that, at clinically achievable concentrations, Tf-Ga potently inhibits T-cell activation and that this immunosuppressive property of gallium may be of adjunctive therapeutic value in the management of disorders characterized by the presence of autoreactive or alloreactive T-cell populations.

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GALLIUM IS A GROUP IIIa metal that bears certain similarities to iron in that it binds avidly to the iron transport protein, transferrin, and can be incorporated into ferritin, the iron storage protein. The cellular uptake of gallium is enhanced by its binding to transferrin. The resultant transferrin-gallium (Tf-Ga) complex blocks cellular iron uptake resulting in iron deprivation and inhibition of cellular proliferation. The antiproliferative activity of Tf-Ga appears to be the result of interference with the activity of ribonucleotide reductase both directly, by interfering with iron incorporation into the iron-containing M2 subunit of the enzyme, and indirectly, by depriving the cell of intracellular iron necessary for ribonucleotide reductase activity.

The ability of gallium to interfere with cellular growth has been an observation that has prompted studies to investigate the therapeutic potential of this agent. Clinical trials have shown gallium to have therapeutic efficacy in lymphoproliferative disorders and urothelial tract tumors. Gallium also inhibits bone resorption and is effective in the treatment of hypercalcemia. In animal models, gallium has been shown to have immunosuppressive activity in autoimmune diseases such as adjuvant-induced arthritis and experimental allergic encephalomyelitis. The efficacy of gallium in autoimmune models, where T cells are thought to play an etiologic role, suggests that gallium may modulate T-cell function. A limited number of studies have shown that gallium can inhibit mitogen-induced proliferative responses, but the overall effect of gallium on T-cell function has not been well defined. In this study, we have investigated the effect of Tf-Ga on human T-cell function and then tested the in vivo immunosuppressive activity of gallium in a murine model of graft-versus-host disease (GVHD) in which alloreactive T cells are necessary for the induction of GVH reactivity.

MATERIALS AND METHODS

Preparation of Tf-Ga. Human transferrin was obtained from Sigma Chemical Co (St Louis, MO). Gallium nitrate was purchased from Alfa Products (Danvers, MA). Tf-Ga (stock solution of 50 mg of protein/mL) was prepared as previously described. Briefly, 3 mol of gallium (as gallium nitrate) was added to each mol of transferrin dissolved in 20 mmol/L acetic acid, and 150 mmol/L NaCl (pH 3.5) buffer. The pH of this solution was raised in gradual increments to 7.4 with 1 mol/L NaHCO3. Saturation of transferrin by gallium (1 mol of transferrin binding to 2 mol of gallium) was confirmed using a Beckman DU 40 spectrophotometer (Beckman Instruments, Fullerton, CA) by measuring the change in absorbance (which occurs with saturation of both metal binding sites of TF) at wavelength 242 nm.

Cell culture. Human venous blood was obtained from normal volunteers. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient centrifugation. Cells were washed and cultured in complete Dulbecco’s modified essential medium (CDMEM) plus 8% human AB serum in either recombinant interleukin-2 (rIL-2) at 500 U/mL (Cetus Corp, Emeryville, CA), phytohemagglutinin-P (PHA-P; Difco Laboratories, Detroit, MI) 5 μg/mL; phorbol 12-myristate 13-acetate (PMA) 1 μg/mL plus ionomycin (0.5 μM); or lymphocyte-conditioned medium (LCM) derived from the supernatant of PBMC cultured in 1% PHA-P for either 24 or 48 hours.

Proliferation assays. Human PBMC were seeded at 10^5 cells/well into triplicate round-bottom 96 well microtiter plates (Costar, Cambridge, MA). Cells were cultured in CDMEM plus 8% AB serum, with and without serial two-fold dilutions of Tf-Ga in the presence of PHA-P, rIL-2, LCM, or PMA plus ionomycin as indicated.
cated. Control wells contained CD26 plus 8% AB serum without any mitogen. In some experiments, addition of PHA-P to cultures containing TF-Ga was delayed as indicated. Cultures were incubated at 37°C in 8% CO₂ for 5 days. Cells were pulsed with 1 μCi/well with tritiated thymidine [³H]-Tdr for the last 18 to 24 hours. Cultures were harvested on an Iototech Cell Harvester (Iototech Biosystems International, Inc, Jeffrey, NY) and the incorporated radioactivity was measured in a Taurus liquid scintillation counter (Micromedic Systems, Inc, Huntsville, AL).

Mixed lymphocyte culture (MLC). PBMC isolated from normal volunteers were used as responder cells in a two-way MLC. Unirradiated PBMC (1 x 10⁶ cells/well) from each of two responders were co-cultured in medium containing serial two-fold dilutions of TF-Ga. Cultures were incubated at 37°C for 6 days and then labeled for the final 18 hours with [³H]-Tdr. Cells were harvested and incorporated radioactivity was measured in a liquid scintillation counter.

Assay for IL-2 production. PBMC were cultured in duplicate in medium plus PMA and ionomycin with or without 2,000 pg/mL TF-Ga. Cultures were incubated for 24 hours at 37°C in 8% CO₂. Control samples consisted of cells only without mitogen or TF-Ga present. The supernatant (150 μL) was harvested, frozen at −80°C, and subsequently thawed before analysis. The activity of IL-2 in culture supernatants was assessed using a radiimmunoassay (Advanced Magnetics, Inc, Cambridge, MA). The percent bound IL-2 was calculated by the formula: (cpm sample − cpm control)/(total cpm) X 100 with total cpm being that due to the ¹²⁵I-IL-2 tracer alone without test samples. The amount of IL-2 in test supernatants was then assessed by interpolation from a standard curve (usually 10%). The percentage of specific ⁵¹Cr release was calculated by the formula: (cpm spontaneous release)/(cpm maximum release − cpm spontaneous release)/(cpm spontaneous release)/(cpm control)/(total cpm) X 100.

Cell mediated lympholysis (CML) assays. PBMC were seeded into 24-well multiplates at a concentration of 5 x 10⁵ cells/well. Cells were grown in CDMEM and 8% AB serum plus 500 U/mL rIL-2 with or without TF-Ga. Cultures were incubated for 3 days at 37°C in 8% CO₂ then harvested, washed, and resuspended in media before use in CML assays. Target cells used in the cytotoxicity assays consisted of the K562 and Daudi cell lines. K562 cells are a natural killer (NK)-sensitive, lymphokine-activated killer (LAK)-sensitive cell line while Daudi cells are NK-resistant, LAK-sensitive. CML assays were performed at eight effector:target (E:T) ratios in triplicate V-bottomed microwells containing 5 x 10⁵ [⁵¹Cr]-labeled targets. The amount of ⁵¹Cr released into the supernatant after 3 hours was determined in a gamma scintillation counter. Maximum ⁵¹Cr-release and spontaneous release values for each target were determined from six to twelve wells containing labeled target cells and 2.5% ‘7X’ detergent (Limbro, McLean, VA) or medium, respectively. Spontaneous release was 20% or less of maximum release (usually 10%). The percentage of specific ⁵¹Cr release was calculated from the mean cpm values of triplicate wells as follows: 100 (cpm experimental − cpm spontaneous release)/(cpm maximum release − cpm spontaneous release).

Flow cytometric analysis. Cell surface marker analysis was performed on PBMC cultured for 3 days in PHA-P using two-color direct immunofluorescence. Cells were washed twice in phosphate-buffered saline plus 2% fetal calf serum and analyzed using standard techniques. Using phycoerythrin or fluorescein isothiocyanate-labeled monoclonal antibodies, cells were stained for CD3/CD4, CD45/CD14, CD3/CD56, CD25/CD3, CD71/CD3 (Becton Dickinson, Mountain View, CA). Appropriate isotypic controls were included. Forward and side scatter properties were used to gate on the lymphocyte population. CD45/CD14 was used to remove red cell and monocyte contamination from the lymphocyte gate. Cells were analyzed on a FACS analyzer (Becton Dickinson) equipped with a FACSlite laser and Consort 30 computer support. The density of CD25 and CD71 on activated T cells was determined by comparing the mean fluorescence intensity in the absence or presence of TF-Ga on CD3+ CD25+ and CD3+ CD71+ cells, respectively, after mitogen stimulation.

RNA isolation and northern blot analysis. Total cellular RNA was isolated from 2.3-5 x 10⁶ cells by the guanidine-thiocyanate-cesium chloride method as previously described.²⁰ RNA samples (20 μg) were subjected to electrophoresis on a 1% agarose gel containing 2.2 mol/L formaldehyde and then transferred to Nitran membranes in 10X SSC using a capillary blotting method. The membranes were washed in 5X SSC, air-dried, and baked at 80°C for 2 hours. The membranes were hybridized for 4 hours at 42°C in 50% formamide, 5X SSPE, 5X Denhardt's solution, 1% dextran sulfate, 1% sodium dodecyl sulfate (SDS), and 200 μg/mL sheared salmon sperm DNA. Membranes were then hybridized overnight to a [³²P]-labeled transferrin receptor cDNA probe (1 x 10⁶ cpm/ml). The membranes were washed three times in 2X SSC at room temperature followed by two 30-minute washes in 2X SSC/0.1% SDS at 65°C. The membranes were exposed to XAR-5 film (Eastman Kodak Co, Rochester, NY) with intensifying screens at −70°C for 24 hours.

Bone marrow transplantation. AKR recipient mice were treated with 1,100 cGy total body irradiation (TBI) within 4 to 8 hours before transplantation. TBI was administered in a single dose using a Shepherd Mark I cesium irradiation (JL Shepherd and Associates, San Fernando, CA). The dose rate was 83.3 cGy/min. Bone marrow (BM) was flushed from donor femurs with CDMEM medium plus 5% fetal bovine serum. The marrow plugs were then washed, resuspended in fresh medium, and counted. Spleens were then washed through sterile mesh screens to obtain single cell suspensions. Spleen cells were then treated with sterile distilled water to eliminate erythrocytes. BM and spleen cells were always greater than 90% viable by Trypan blue dye exclusion. Irradiated recipient mice received a single intravenous injection containing 10 x 10⁶ BM cells with or without 20 x 10⁶ spleen cells.

In vivo administration of gallium. For continuous subcutaneous delivery of citrate-buffered gallium nitrate, a 14 day mini-osmotic pump (ALZA Corporation, Palo Alto, CA) was implanted in the backs of experimental mice under general anesthesia. GVHD control animals received pumps containing equivalent volumes (200 μL) of phosphate-buffered saline. The total dose of gallium administered to animals over 14 days was either 37 mg/kg, 49 mg/kg, or 65 mg/kg. These doses were selected based on prior studies that had demonstrated a total dose of 63 mg/kg to be the LD₅₀ and 80 mg/kg to be the LD₉₀ in mice receiving daily intraperitoneal injections of gallium for a total of 10 days.²¹ The mean weights of experimental animals in each group before transplant were determined and the concentration of gallium in individual pumps modified as needed in each experiment to ensure that the total dose administered to recipients was the same in replicate experiments. Pumps were routinely removed in all animals after 14 days, and inspected to ensure that the entire volume had been expelled.

Experimental design. AKR recipients were administered 1,100 cGy TBI as pretransplant conditioning. Based on the fact that 18 of 100% irradiated control mice (without marrow transplantation) died at a median of 10.5 days after irradiation (range 5-14 days), this was considered a lethal dose. Animals were transplanted with 10 x 10⁶ cells) with or without 20 x 10⁶ of spleen.
cells. Irradiated mice receiving BM do not develop GVHD because of an insufficient number of T cells in the BM inoculum (typically 2% to 3%).

Statistical analysis. The fluorescence intensity of CD25 and CD71 expression on mitogen-stimulated CD3+ T cells was expressed as the mean of five individual experiments ± one standard deviation. Experimental groups were compared using the paired Student's t test. The median survival of gallium-treated and GVHD control mice was compared using the Wilcoxon-Rank Sum Test. A two-tailed P value < .05 was considered significant.

RESULTS

Tf-Ga inhibits polyclonal and alloantigen-induced T-cell proliferation. PBMC were stimulated by incubation with either PHA, LCM, rIL-2, or PMA plus ionomycin in the presence of 0 to 2,000 μg/mL of Tf-Ga. Addition of Tf-Ga produced a dose-dependent inhibition of proliferation regardless of the mitogenic stimulus (Fig 1). Lower concentrations of Tf-Ga were required for significant inhibition (>90%) when PHA or PMA/ionomycin were used rather than when IL-2 or LCM were used to polyclonally activate PBMC (Table 1). When cells were cultured in the presence of high concentrations of rIL-2 or LCM, a concentration in excess of 2,000 μg/mL of Tf-Ga was required for 90% inhibition. A threshold concentration of at least 500 μg/mL of Tf-Ga was required for appreciable inhibition in all instances except when PMA/ionomycin was used as a mitogen.

The effect of Tf-Ga on antigen-specific proliferation was then examined in a bidirectional MLC assay. The results of three independent experiments are shown in Table 2. At a concentration of 2,000 μg/mL Tf-Ga inhibited proliferation by approximately 90%. As observed with polyclonal stimuli, little inhibition was observed below a concentration of 500 μg/mL of Tf-Ga in all instances except when PMA/ionomycin was used as a mitogen.

Table 1. Inhibitory Concentration of Tf-Ga Required for Suppression of Mitogenic Responses

<table>
<thead>
<tr>
<th>Mitogenic Stimulus</th>
<th>No. of Experiments</th>
<th>IC50 (μg/mL)</th>
<th>IC90 (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>3</td>
<td>2,070 ± 926</td>
<td>13,831 ± 10,407</td>
</tr>
<tr>
<td>LCM</td>
<td>3</td>
<td>1,216 ± 131</td>
<td>2,649 ± 316</td>
</tr>
<tr>
<td>PHA</td>
<td>2</td>
<td>709 ± 57</td>
<td>1,709 ± 66</td>
</tr>
<tr>
<td>PMA/ionomycin</td>
<td>3</td>
<td>111 ± 27</td>
<td>797 ± 177</td>
</tr>
</tbody>
</table>

PBMC were cultured in media plus either IL-2, LCM, PHA, or PMA plus ionomycin as indicated in Materials and Methods. The concentration of Tf-Ga at which proliferation, as assessed by thymidine incorporation, was 50% (IC50) or 90% (IC90) inhibited was calculated by fitting a linear regression curve to the data. Data are presented as mean ± 1 standard deviation.
Table 2. Effect of Ti-Ga on the MLC Response

<table>
<thead>
<tr>
<th>Concentration of Ti-Ga (µg/mL)</th>
<th>MLC Reactivity (cpm ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>36,386 ± 4,852</td>
</tr>
<tr>
<td>31.2</td>
<td>34,060 ± 1,670</td>
</tr>
<tr>
<td>62.5</td>
<td>32,814 ± 5,225</td>
</tr>
<tr>
<td>125</td>
<td>31,845 ± 5,249</td>
</tr>
<tr>
<td>250</td>
<td>29,496 ± 2,884</td>
</tr>
<tr>
<td>500</td>
<td>28,412 ± 540</td>
</tr>
<tr>
<td>1,000</td>
<td>27,253 ± 502</td>
</tr>
<tr>
<td>2,000</td>
<td>2,771 ± 450</td>
</tr>
</tbody>
</table>

PBMC obtained from normal nonrelated donors were seeded at a concentration of 1 × 10⁶ cells/well and cocultured for 6 days in medium alone or medium plus the designated concentration of Ti-Ga. The degree of proliferation was assessed by thymidine incorporation. Data are presented as the mean ± 1 standard deviation of triplicate determinations.

Table 3. Prior Exposure to Ti-Ga Does Not Prevent Subsequent Mitogen-Induced T-Cell Proliferation

<table>
<thead>
<tr>
<th>Culture Conditions</th>
<th>³H-Tdr Incorporation (cpm ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media (3 d)</td>
<td>70 ± 49</td>
</tr>
<tr>
<td>PHA (3 d)</td>
<td>72,860 ± 932</td>
</tr>
<tr>
<td>PHA + Ti-Ga (3 d)</td>
<td>8014 ± 227</td>
</tr>
<tr>
<td>Media (1 d) + PHA</td>
<td>56,295 ± 6,456</td>
</tr>
<tr>
<td>Ti-Ga (1 d) + PHA</td>
<td>47,533 ± 7,277</td>
</tr>
<tr>
<td>Media (2 d) + PHA</td>
<td>71,885 ± 2,665</td>
</tr>
<tr>
<td>Ti-Ga (2 d) + PHA</td>
<td>66,194 ± 4,178</td>
</tr>
<tr>
<td>Media (3 d) + PHA</td>
<td>64,617 ± 8,520</td>
</tr>
<tr>
<td>Ti-Ga (3 d) + PHA</td>
<td>34,448 ± 4,349</td>
</tr>
</tbody>
</table>

PBMC were seeded at 1 × 10⁶ cells per well in CDMEM plus 8% AB serum alone or medium plus Ti-Ga (2,000 µg/mL) for 1 to 3 days. Cells were then washed and fresh medium containing PHA (5 µg/mL) was added for an additional 3 days. The degree of proliferation after 4 to 6 days in culture was then assessed by thymidine incorporation. Data are presented as the mean ± 1 standard deviation of quadruplicate determinations.

µg/mL, indicating that blockade of MLC reactivity was concentration dependent.

Inhibition of T-cell proliferation by Ti-Ga is time dependent. Because Ti-Ga could inhibit mitogen and alloantigen-induced T-cell proliferation when added at the onset of culture, studies were conducted to determine if Ti-Ga could inhibit proliferation after T cells had been exposed to mitogen. PBMC were stimulated with PHA, and Ti-Ga was then added to individual wells at 0, 1, 2, or 3 days of culture. A concentration of 2,000 µg/mL, which had been shown to effectively suppress PHA-induced proliferative responses (Fig 1), was used. Addition of Ti-Ga within 24 hours after initiation of culture resulted in greater than 60% suppression of the proliferative response (Fig 2). In contrast, Ti-Ga had little effect on PHA-induced proliferation when added 2 or 3 days after culture initiation, indicating that activated T cells were less amenable to inhibition by Ti-Ga.

We then performed studies to determine whether exposure to Ti-Ga resulted in irreversible inhibition of T cell proliferation. PBMC were exposed to Ti-Ga (2,000 µg/mL) for 1, 2, or 3 days, washed, and then stimulated with fresh PHA for 3 additional days to assess the proliferative response in the absence of gallium. Control wells consisted of PBMC cultured in medium alone for 1 to 3 days followed by a 3-day exposure to PHA. Prior treatment with Ti-Ga for 1 to 3 days did not prevent subsequent T-cell proliferation to mitogen (Table 3), indicating that T-cell proliferation is not irreversibly inhibited by Ti-Ga.

Ti-Ga does not inhibit the secretion of IL-2. T-cell proliferation is dependent on the expression of IL-2 receptors on the T-cell surface and the synthesis of IL-2. We therefore performed studies to determine whether Ti-Ga inhibited T cell proliferation by interfering with either of these two events. The effect of Ti-Ga on IL-2 secretion was investigated using a radioimmunoassay. PBMC were stimulated for 24 hours by PMA and ionomycin with or without 2,000 µg/mL Ti-Ga. Control wells contained medium only without Ti-Ga. There was no significant difference in IL-2 secretion between PMA and ionomycin-stimulated cultures in the presence or absence of Ti-Ga (P = .41; Table 4), indicating that Ti-Ga did not inhibit IL-2 secretion by T cells.

Ti-Ga decreases the density of IL-2 receptors (CD25) on activated T cells. We then assessed whether Ti-Ga altered the expression of CD25 on activated T cells. PBMC stimulated with PHA in the presence or absence of 2,000 µg/mL Ti-Ga were analyzed by flow cytometry for expression of CD25. The percentage of T cells expressing CD25 was reduced in the presence of Ti-Ga (P = .01; Table 5). To determine whether Ti-Ga affected the density of CD25 expression on activated T cells, we assessed the fluorescence.
expression in mitogen-stimulated T cells. PBMC were cultured for 24 hours in medium alone, medium plus PMA/ionomycin, or medium, PMA/ionomycin, and 2,000 µg/mL Tf-Ga. The supernatant was then analyzed for IL-2 by radioimmunoassay. Data in individual experiments are presented as the mean of duplicate determinations.

Table 4. Effect of Tf-Ga on IL-2 Production

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
<th>Average ± SD (pg/100 µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>6.3</td>
<td>7.3</td>
<td>7.9</td>
<td>7.2 ± 0.8</td>
</tr>
<tr>
<td>PMA</td>
<td>275.8</td>
<td>292.1</td>
<td>263.0</td>
<td>277.0 ± 14.6</td>
</tr>
<tr>
<td>PMA + Tf-Ga</td>
<td>265.6</td>
<td>251.2</td>
<td>269.5</td>
<td>262.1 ± 8.6</td>
</tr>
</tbody>
</table>

PBMC were cultured for 24 hours in medium alone, medium plus PMA/ionomycin, or medium, PMA/ionomycin, and 2,000 µg/mL Tf-Ga. The supernatant was then analyzed for IL-2 by radioimmunoassay. Data in individual experiments are presented as the mean of duplicate determinations.

Table 5. Effect of Tf-Ga on the Percentage of Mitogen-Stimulated CD3⁺ T Cells Expressing CD25 and CD71

<table>
<thead>
<tr>
<th>Mitogen</th>
<th>Tf-Ga (µg/mL)</th>
<th>% CD3⁺ T Cells Coexpressing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD25</td>
<td>CD71</td>
</tr>
<tr>
<td>None</td>
<td>—</td>
<td>2 ± 0.9</td>
</tr>
<tr>
<td>PHA</td>
<td>—</td>
<td>93 ± 2.6</td>
</tr>
<tr>
<td>PHA</td>
<td>2,000</td>
<td>81 ± 4.8</td>
</tr>
</tbody>
</table>

PBMC were cultured for 3 days in medium alone, medium plus PHA, or medium plus PHA and 2,000 µg/mL Tf-Ga. Cells within the lymphocyte gate were then analyzed by flow cytometry for surface expression of CD3, CD25, and CD71. Data are presented as the mean ± 1 standard deviation of five individual experiments.

Intensity of CD25 expression on CD3⁺ CD25⁺ cells after two-color staining. The mean fluorescence intensity of CD25 expression was 652 ± 179 in the absence of Tf-Ga and 386 ± 80 in the presence of Tf-Ga (mean of five experiments) (P < .005), indicating that Tf-Ga decreased the density of IL-2 receptors on activated T cells.

Tf-Ga produces an upregulation of the transferrin receptor (CD71) in activated T cells. Because cellular proliferation is linked to transferrin receptor expression, we assessed the effect of gallium on transferrin receptor expression in mitogen-stimulated T cells. PBMC were cultured for 3 days in PHA with or without Tf-Ga and the percentage of CD3⁺ CD71⁺ cells was evaluated by flow cytometry. As expected, resting T cells did not express a significant level of CD71 (Table 4). Addition of Tf-Ga to PHA-stimulated cultures did not increase the percentage of T cells that expressed CD71 when compared with mitogen-stimulated cultures alone (P = .19). However, the density of CD71 antigen expression on CD3⁺ T cells as assessed by fluorescence intensity was augmented in the presence of Tf-Ga (155 ± 38 without Tf-Ga, and 332 ± 143 with Tf-Ga, mean of five experiments) (P = .023). To assess whether transferrin receptor mRNA was increased in T cells exposed to gallium, RNA was extracted from PHA-stimulated PBMC cultured in the presence or absence 2,000 µg/mL of Tf-Ga for 3 or 6 days and hybridized with a transferrin receptor cDNA probe in a northern blot assay. Each lane was loaded with 20 µg of RNA. The same filter was hybridized with the β-actin cDNA probe to permit normalization of RNA loading. Data are from one of three representative experiments.

![Fig 3. Effect of Tf-Ga on transferrin receptor mRNA in mitogen-stimulated PBMC](image)

3 and 6 days, while levels remained elevated in cell cultures exposed to Tf-Ga. Collectively, these data indicate that Tf-Ga upregulates the transferrin receptor on mitogen-stimulated T cells.

Tf-Ga does not inhibit the induction of LAK activity. The ability of gallium to modulate lytic activity was assessed by examining the effect of Tf-Ga on the induction of LAK activity by IL-2. PBMC cultured for 3 days in high dose IL-2 with or without Tf-Ga were tested in a chromium release assay against K562 and Daudi cell targets. Lytic activity against both target cell populations was unaffected by concentrations of Tf-Ga ranging from 500 to 2,000 µg/mL at all E:T ratios tested (Fig 4). Thus, at concentrations that effectively inhibited T-cell proliferation, LAK activity was retained.

Gallium prolongs survival of mice undergoing severe GVHD. To assess the immunosuppressive action of gallium in vivo, we investigated the effect of gallium in a murine model of GVHD where donor (B10.BR, H-2b) and recipient (AKR/J, H-2a) are MHC-matched but differ at multiple minor histocompatibility antigens. In this model,
MODULATION OF T-CELL FUNCTION BY GALLIUM

Fig 4. Effect of Tf-Ga on the induction of LAK activity. PBMC were cultured for 3 days in medium plus 500 μg/ml IL-2 alone (□) or in the presence of 500 μg/ml Tf-Ga (■), 1,000 μg/ml Tf-Ga (■■), or 2,000 μg/ml Tf-Ga (■■■). Cells were then tested in a chromium release assay against K562 or Daudi targets. Data shown are from one of six representative experiments at the four highest E:T ratios tested.

the development of GVHD is dependent on the presence of alloreactive mature donor T cells.24 Gallium was administered subcutaneously by continuous infusion to maintain steady-state levels, since in vitro studies (Tables 1 and 2) had demonstrated that inhibition of T-cell proliferation was concentration dependent. AKR mice transplanted with B10.BR only (n = 18) did not develop GVHD and 83% (15/18) survived at least 60 days posttransplant. In contrast, AKR recipients (n = 30) transplanted with B10.BR BM plus 20 × 10⁶ spleen cells underwent a severe GVH reaction and all animals died within 40 days of transplant (median survival, 16 days; Table 6). Administration of gallium by continuous infusion resulted in a modest but statistically significant prolongation of survival (median survival, 27 days, P = .00001) with 20% (10/50) of mice surviving past 40 days and 6% surviving to the conclusion of the experiment (day 63). The three surviving mice were all treated at a dose of 49 mg/kg. All mice had clinical evidence of GVHD indicating that gallium, at the doses studied, was able to ameliorate but not abrogate GVH reactivity. While gallium had efficacy at all dose levels, survival was most significantly prolonged at the highest dosage tested (55 mg/kg).

DISCUSSION

Whereas the antineoplastic activity of gallium has been examined previously in several studies, its action on the
immune system has not been well characterized. We therefore undertook this investigation to examine the in vitro and in vivo effects of gallium on T-cell function. Our studies show that Tf-Ga is a potent inhibitor of polyclonal and alloantigen-induced human T-cell proliferation. Proliferation induced by all mitogens tested was significantly reduced in the presence of Tf-Ga in a dose-dependent fashion and Tf-Ga most effectively inhibited proliferation induced by PHA and PMA/ionomycin with IC₅₀ doses less than 2,000 μg/mL. In contrast, proliferation induced by LCM and rIL-2 was less efficiently inhibited by Tf-Ga, possibly due to the fact that these mitogens also stimulate non-T-cell populations (eg, NK and B cells), which may be more resistant to gallium. Although Tf-Ga was most effective as an inhibitor when present at the time of initial exposure of cells to mitogens, it was relatively ineffective when added after T-cell activation had been established. This suggests that Tf-Ga acts by interfering with early steps in T-cell activation.

The activation of T cells by mitogens results in the induction of a series of gene products that are essential for the subsequent proliferation of these cells. Studies have shown that the synthesis of IL-2 and its receptor is followed by the appearance of the transferrin receptor and that antibody to the IL-2 receptor (anti-TAC) blocks transferrin receptor expression on PHA-activation lymphocytes. Conversely, transferrin receptor mRNA transcription has also been shown to precede that of the IL-2 receptor when T cells are activated with phorbol esters and ionophores, suggesting that the relationship of IL-2 and transferrin receptor expression may depend on culture conditions. Because of the important role of these proteins in T-cell activation, we examined the effect of Tf-Ga on IL-2 secretion and the expression of transferrin and IL-2 receptors. Although Tf-Ga did not impair the secretion of IL-2 by T cells, it did significantly reduce the density of IL-2 receptors (CD25 antigen) on these cells. Because this decrease in IL-2 receptor was accompanied by only a minor reduction in actual number of CD25⁺ T cells, it suggests that Tf-Ga produces a decrease in the synthesis of IL-2 receptor. In contrast to its effect on the IL-2 receptor, Tf-Ga produced an increase in transferrin receptor mRNA and protein expression in activated T cells.

The divergent effect of Tf-Ga on the expression of IL-2 and transferrin receptors resembles that seen with iron chelators. Carotenuto et al have shown that iron chelation by deferoxamine decreases a decrease in the expression of IL-2 receptors on activated T cells without inhibiting IL-2 secretion, while others have shown that cellular iron deprivation leads to an increase in the synthesis of transferrin receptors. The basis for the iron deprivation-induced decrease of IL-2 receptors is unknown; however, the upregulation of transferrin receptors is due to an increase in the binding of cytoplasmic iron regulatory proteins (IRPs) to transferrin receptor mRNA. This, in turn, stabilizes transferrin receptor mRNA and increases its translation. Our earlier studies in leukemic HL60 cells have shown that Tf-Ga blocks transferrin receptor-mediated iron uptake, producing cellular iron deprivation and an increase in transferrin receptor mRNA and protein. The Tf-Ga-induced increase in transferrin receptor mRNA is due to stabilization of this mRNA rather than an increase in its transcription, a finding that is consistent with an increase in the activity of IRPs (increased mRNA binding). Although we did not examine IRP-mRNA interactions in the present study, it appears reasonable to conclude that the Tf-Ga-induced increase in transferrin receptors in activated T cells is mediated by this mechanism. It should be appreciated, however, that the action of gallium most likely extends beyond induction of cellular iron deficiency since gallium per se may perturb cell function by directly interacting with intracellular macromolecules. Studies are in progress to identify such interactions.

The therapeutic value of gallium has been exploited in several animal models in which autoreactive T cells are thought to play an etiological role. Matkovic et al and Whitacre et al have demonstrated that weekly injections of gallium nitrate prevented clinical and biological changes associated with experimental allergic encephalomyelitis and adjuvant arthritis in rodent models. In both instances, animals treated in vivo with gallium had reduced purified protein derivative-specific lymphocyte proliferative responses in vitro. To determine whether gallium could inhibit autoreactive T cells, we examined the effect of gallium in a murine model of GVHD. In this model, lethally irradiated AKR recipients develop GVHD when supplemental donor splenic T cells are added to the BM graft. Since in vitro studies have demonstrated that inhibition of polyclonal and alloantigen T-cell responses by Tf-Ga was concentration dependent, we administered gallium by continuous infusion to maintain steady-state levels. We observed that gallium was able to prolong median survival in mice by approximately 70%, but was not able to abrogate GVH reactivity as most animals still died of GVHD. We postulate that the salutary effect of gallium was most likely due to inhibition of donor T-cell proliferation during the interval that T cells were exposed to gallium. Incomplete protection may have been due to several factors. First of all, gallium was administered for only 14 days; therefore, clonal expansion of T cells could have occurred once gallium treatment was withdrawn. This is consistent with in vitro data (Table 3), which showed that T cells were fully capable of proliferating once they were removed from Tf-Ga and then exposed to mitogen. Second, since inhibition of T-cell proliferation is critically dependent on the concentration of gallium, it is possible that the doses we used were suboptimal. We selected the dosage range based on prior studies that had demonstrated increased lethality when bolus dosing in excess of 63 mg/kg was used. However, since continuous infusion of gallium has been associated with less toxicity than bolus administration, it is conceivable that higher doses would have been tolerated. Moreover, gallium toxicity can also be minimized by hydration, which has been shown to mitigate gallium-induced nephrotoxicity. Because vigorous hydration of mice is technically more cumbersome than in humans, this variable was difficult to assess. Nonetheless, it is possible that administration of higher doses of gallium in association with hydration would allow for an augmented therapeutic index.

The clinical feasibility of using gallium is supported by
data indicating that the concentration of Tf-Ga necessary for T-cell immunosuppression is likely to be clinically achievable, since significant proliferative inhibition was observed with concentrations of 1,000 to 2,000 μg/mL. Given that only one third of circulating transferrin (1.7 to 3.9 mg/dL) is typically bound to iron, a significant amount is potentially available for binding to gallium. Saturation of these remaining sites would allow concentrations of Tf-Ga to be achieved, which have been shown to inhibit T-cell responsiveness in vitro. While gallium alone is unlikely to be sufficient to prevent autoreactive and alloreactive T-cell proliferation in vivo, this agent may be of value in association with other therapeutic modalities. A potential advantage of gallium relative to other modalities used in these disorders is that gallium targets only cells expressing the transferrin receptor, which must be expressed before T cells are activated and can proliferate. Once within the cell, gallium is also capable of directly interacting with macromolecules essential for cellular proliferation and function such as ribonucleotide reductase. Thus gallium has distinct advantages over the use of iron chelators such as desferoxamine that act nonspecifically. The optimal schedule and route of administration of gallium to achieve these effects is unknown, but further study is warranted to determine whether gallium has a role in the treatment of autoimmune disorders or conditions such as GVHD in which T-cell alloreactivity is the major inciting pathophysiological event.

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Modulation of in vitro and in vivo T-cell responses by transferrin-gallium and gallium nitrate

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