Uptake of Gallium-67 in Transfected Cells and Tumors Absent or Enriched in the Transferrin Receptor

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Gallium-67 has been a controversial tumor-imaging agent in nuclear medicine for decades. This controversy centers on why tumors are variable in gallium-avidity, whether ^{67}Ga uptake is a transferrin-independent or dependent process, and whether tumors and normal tissues differ in mechanism of uptake. If the factors that control uptake of ^{67}Ga were understood better, then efforts to improve oncologic imaging with ^{67}Ga by increasing the tumor activity, or by decreasing the background, may be warranted. **Methods:** Conventional systems for evaluating the mechanism and control of ^{67}Ga uptake have significant limitations. We have endeavored to circumvent these by developing a pair of transfected cell lines. One cell line has no transferrin receptor. In the other, the human transferrin receptor has been restored by transfection and is over-expressed constitutively, without the necessity to manipulate factors such as cell growth or iron content. The uptake of ^{67}Ga, both as a citrate salt and as a gallium-transferrin complex, was examined in these pairs of cells in vitro. The effect of calcium and of soluble (ionic) iron concentration on ^{67}Ga uptake also was determined. Tumors were grown as explants of these cells in nude mice and comparisons of uptake of ^{67}Ga by these tumors in vivo were made. **Results:** The in vivo uptake of ^{67}Ga is significantly increased in tumors in which the transferrin receptor is overexpressed, compared to those without a functional transferrin receptor. However, a notable amount of accumulation of ^{67}Ga also occurs, both in vitro and in vivo, by a transferrin-independent route. In vitro experiments demonstrate that the uptake of ^{67}Ga by the transferrin-independent route can be enhanced further to levels that equal or exceed those achieved by the transferrin-dependent route by increasing the content of calcium or iron salts in the incubation medium. **Conclusion:** Significant transferrin-independent uptake of ^{67}Ga occurs both in vitro and in vivo. This uptake can be stimulated further in vitro, suggesting that in vivo enhancement might also be possible to enhance the utility of the radiometal for tumor imaging.

Key Words: gallium-67; neoplasms; mouse; transferrin; transfection; transferrin receptor

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In 1969, Edwards and Hayes first described the accumulation of ^{67}Ga in the lymph nodes of a patient with Hodgkin's disease (*1*). Subsequently, ^{67}Ga has been shown to localize in a variety of tumors (*2,3*). In nuclear medicine, the utility of ^{67}Ga as a tumor-imaging agent is variable. Many types of tumors accumulate gallium poorly. Others, such as hepatomas and lymphomas, tend to be gallium avid but can be inconsistent in magnitude and uniformity of uptake. Nonetheless, no other gamma-emitting radiopharmaceutical, including expensive monoclonal antibodies and receptor-avid agents, has surpassed ^{67}Ga in cost-effectiveness, availability and use for general tumor imaging.

Despite years of experience in imaging with the radiometal, the precise mechanism by which ^{67}Ga accumulates in tumors and normal tissues remains controversial. The most popular theory is that gallium, as an analog of iron, is taken up by the transferrin receptor (TfR) as a gallium-transferrin (Ga-Tf) complex (*4*). Tumors may be gallium-avid if they are enriched in the TfR. That gallium can enter tumor cells as a Ga-Tf complex is well supported by competition experiments with Tf-Fe and by blocking the TfR with antibodies (*5–8*). Despite these observations, there is significant evidence that mechanisms other than the TfR may be responsible for the uptake of ^{67}Ga in tumors (*9–12*). Gallium may dissociate from Tf in the acidic extracellular environment of tumors (*13–15*). There is a poor correlation between TfR density and the degree of tumor uptake of gallium (*9,16–18*). Uptake of gallium also continues to a significant degree in the absence of Tf, or when TfR binding sites are blocked with an antibody (*9,18,19*). During iron overload (when TfR expression is down-regulated), uptake of gallium in many tumors is unchanged. When tumor-bearing rats are rendered iron deficient (which increases TfR's in many tissues), uptake of ^{67}Ga is increased in other tissues but remains the same in tumors (*20*). When Tf binding sites are saturated with iron or scandium after administration of ^{67}Ga, uptake of gallium in tumors, relative to normal tissues, can actually increase (*21,22*). The fact that tumors and normal tissues may accumulate ^{67}Ga by a different mechanism has important practical significance for imaging. If true, then efforts to manipulate the uptake of ^{67}Ga, either by reducing uptake in background tissues or enhancing uptake in tumors, might be feasible.

Transferrin-independent methods for iron uptake have been suggested both in vitro and in vivo (*23–27*). One route for Tf-independent iron uptake has been characterized (*25,26*). Gallium and iron may share a Tf-independent route of uptake (*8,10*). Uptake of iron by the Tf-independent pathway is stimulated by iron salts and gallium nitrate. Ferric chloride also stimulates uptake of gallium nitrate. Neither effect is diminished by an anti-TfR monoclonal antibody (*8*).

We previously compared the uptake of ^{67}Ga by two types of implanted tumors and by normal tissues in normal and severely hypotransferrinemic strains of Balb/C mice (*28*). Although uptake of ^{67}Ga by normal soft tissues was markedly suppressed in the hypotransferrinemic mouse, uptake by bone and an implantable myeloma tumor was maintained, suggesting that

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uptake of ^{67}Ga by bone and some tumors may be a Tf-independent process.

Our observations in hypotransferrinemic mice have limitations. Transferrin is not absent, but only severely diminished in hypotransferrinemic mice. Since the hypotransferrinemic mice are very anemic, transferrin receptor expression could be increased significantly in tumor explants, concentrating ^{67}Ga (relative to other tissues) even if only small amounts of Ga-Tf are present. An additional limitation of our murine study (and of all in vitro experiments where there is attempted removal of Tf from the system) is that the uptake of ^{67}Ga by cells could be mediated by Tf synthesized and secreted locally by the tumor cells.

To study the relative contributions of Tf-dependent and Tf-independent factors on the uptake of ^{67}Ga, many have attempted to manipulate expression of the TfR or to block the receptor with an antibody. These approaches also have limitations. To alter expression of the TfR, it is necessary to either subject animals or cells to iron depletion/repletion, or to alter differentiation and cellular proliferation by exposure to specific mitogens or cytokines (*29,30*). These maneuvers may alter the organism physiologically in many ways that confound a well-controlled experiment. Our own experience agrees with the published data of many others (*6,19*), that monoclonal antibodies directed against the TfR are only partially successful in blocking the uptake of ^{67}Ga. This persistent uptake in the presence of monoclonal antibodies may be due to incomplete blocking by the antibody, or to an alternate Tf-independent route of uptake. An alternative to existing biological systems is needed to allow well-controlled studies of the mechanisms for ^{67}Ga uptake.

To circumvent the above limitations, we have examined the uptake of ^{67}Ga both in vitro and in vivo in two novel cell lines. One cell line has no TfR. In the other, the human TfR has been restored by transfection and is over-expressed constitutively, without the necessity to manipulate factors such as cell growth or iron content.

MATERIALS AND METHODS

Construction of Plasmids

The plasmid pEDmtxTfR was constructed by a series of endonuclease digestions and ligations, summarized in Figure 1. The cDNA for the human TfR was taken from a plasmid constructed by McClelland et al. (*31*), pCDTR-1 (Genetics Institute, Cambridge, MA). This cDNA has been used by others to express a normally-functioning TfR (*32*). The cDNA in pCDTR-1 is flanked by both the 5′ and 3′ untranslated regions taken from the chromosomal gene for the TfR that regulate expression of the TfR in response to growth, mitogenic stimulation and iron depletion (*30,33*). We subcloned only the coding sequences of the TfR cDNA, and not the 5′ and 3′ untranslated regions, into an eukaryotic expression vector designed to result in the constitutive expression and amplification of the transfected genes.

The recipient expression vector for the TfR cDNA was pEDMTXr, provided to us by Kaufman et al. (*34*). In the resultant plasmid, called pEDmtxTFR, the modified human TfR cDNA is driven by two tandem constitutive viral promoters and is the upstream component of a bicistronic message. The downstream cistron is the high-Km dihydrofolate reductase (DHFR) gene, which confers resistance to methotrexate (MTX). Selection for resistance to high concentrations of MTX also will result in augmented expression of the TfR cDNA (*35*). An encephalomyocarditis virus (EMCV) site for ribosomal reinitiation is located between the inserted cDNA and the DHFR sequences. It reduces the selective pressure to develop deletional mutations in the upstream cistron by promoting greater efficiency in expression of the downstream cistron (*36*). As a control plasmid, we used the Kaufman plasmid, pEDMTXr, which is identical to pEDmtxTFR except that the inserted TfR cDNA coding sequences are absent.

Transfection and Selection of TfR+ and TfR− Cells

A mutant Chinese hamster ovary (CHO) cell line, TRVb, which does not express the TfR, was transfected with either pEDmtxTFR or the control plasmid, pEDMTXr. TRVb cells were provided to us by their originator, McGraw at Columbia University. McGraw et al. (*32*). mutagenized these cells chemically and selected for resistance to a transferrin-diptheria toxin conjugate. TRVb cells, although lacking their own TfR gene, are capable of expressing a functional TfR when it is supplied by transfection. The plasmids were transfected into recipient TRVb cells by electroporation, using the protocol of the Genetics Institute (Cambridge, MA) for CHO cells. All cells were co-transfected with a second plasmid, pHS1SVneoB, from Chiron (Emeryville, CA) (*37*). This plasmid confers resistance to the toxic aminoglycoside G-418. In a mixture of electroporated plasmids, the molar ratio of G-418-resistant plasmids to primary plasmids was 1:20 so that transfectants surviving initial selection in G-418 would be likely to have assimilated the primary plasmid as well.

Transfected cells initially were selected in complete medium containing 0.573 mg/ml G-418 (Geneticin sulfate, Gibco BRL, Cleveland, OH). Resistant colonies were cloned by limiting dilution in 96-well dishes. Amplification of expression by clones of successful transfectants was accomplished by requiring the cells to survive exposure to incrementally increasing concentrations of methotrexate, a process which took approximately 1 yr. At the conclusion of selection, cells were again subcloned by limiting dilution. The resultant cells are resistant to 1000 μM MTX, a concentration that is 4000-fold greater than can be survived by amplification of the endogenous DHFR gene by either wild-type WTB cells and untransfected TRVb cells.

The TRVb cells with the restored human TfR cDNA (minus the iron regulatory elements) are named TfR+ cells. Control cells, (TfR- cells), received the control plasmid (pEDMTXr) without the TfR cDNA.

General Procedures

Cell Culture. Cells were cultured in monolayer at 37°C, 8% CO_2, in Dulbecco's Modified Essential Medium (DMEM) with 10% HyClone Supplemented Calf Serum (HyClone, Logan, UT), and 1000 uM MTX (Lederle, Carolina, PR) to maintain amplification of the transfected cDNAs.

Separation of Cells from Radioactive Contaminants After Incubations with Radioactive Substrates. After incubation of cells with all radioactive substrates described, the flasks were immediately placed on ice. The radioactive material was removed and the monolayers were washed three times with 5 ml each ice cold Hank's Balanced Salt Solution (HBSS). Cells were washed once with phosphate-buffered saline (PBS), warmed to 37°C. The PBS was removed, and cells were overlaid with 1.5 ml of 0.25% trypsin, 1 m*M* EDTA. The trypsin was removed immediately and the cells were incubated briefly (1 min) at 37°. The trypsinized cells from each flask were collected in 200 μl ice cold PBS.

By our modification of a method previously published for the radiolabeling of protozoan parasites (*38*), the washed and trypsinized cells were separated from unbound contaminating radioactivity. The 200 μl of cells in PBS were layered gently over 200 μl of an 8.5:1.5 ratio of dibutylpthalate-to-liquid paraffin oil in a 1.5-ml microfuge tube. With care not to agitate the mixture, the tubes were centrifuged at 12,000 rpm for 2 min in a microcentrifuge. The supernatant and the oil were removed carefully from the top of the tube by a Pasteur pipette attached to water suction. The

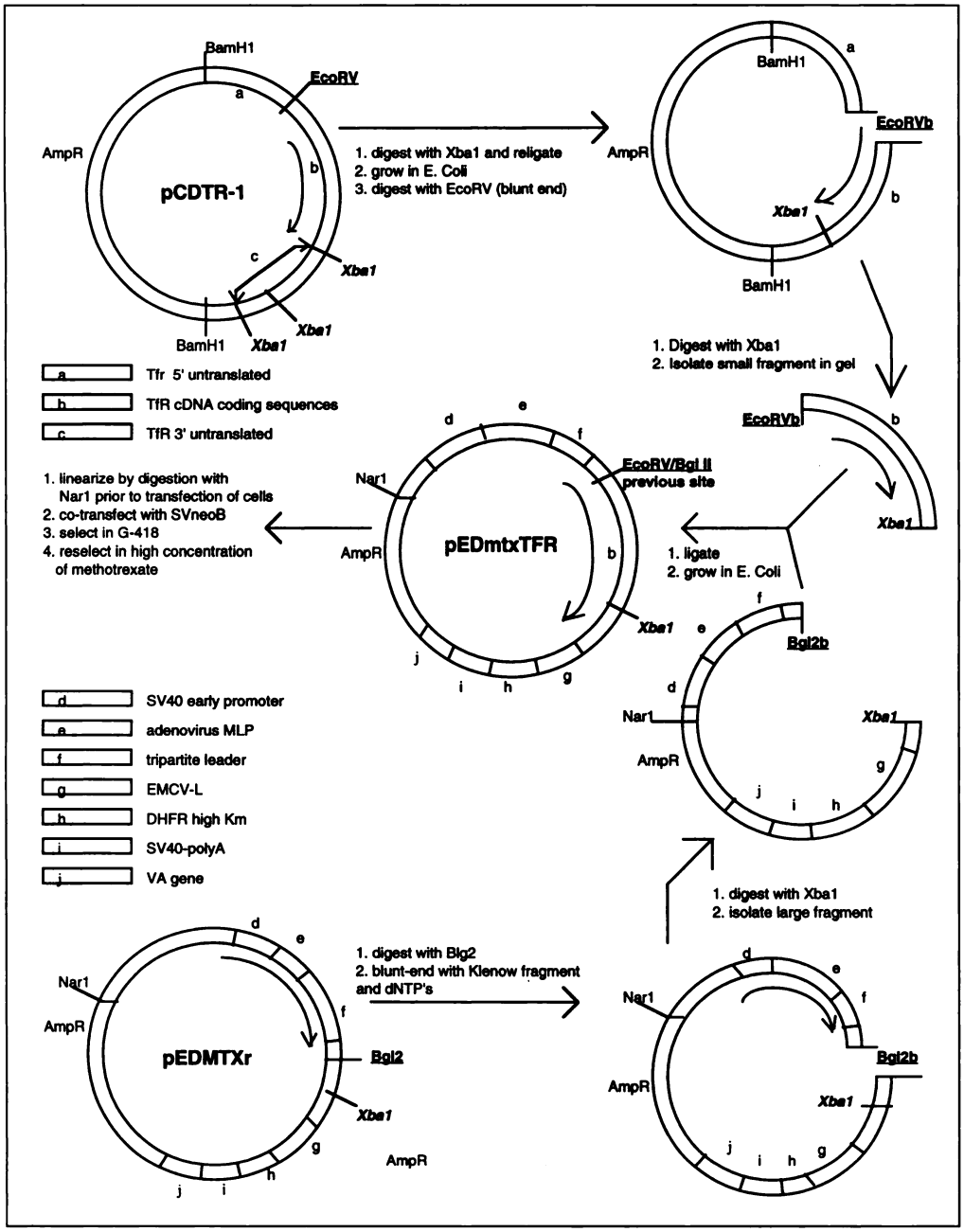

FIGURE 1. Construction and transfection procedure for the plasmid pEDmtxTFR. The coding sequences were isolated from the 5′ and 3′ untranslated regulatory elements for the human transferrin receptor from the plasmid pCDTR-1. The cDNA coding sequences were subcloned into a second eukaryotic expression vector (pEDMTXr), designed to constitutively amplify expression of the inserted cDNA.

bottom of the microfuge tube, containing the cell pellet (typical pellet volume ~100 μl), was then clipped with a microfuge tube cutter into a counting vial containing 900 μl of a solution of 200 m*M* NaOH, 1% SDS. The cell pellet was dissolved in this solution.

Protein Assays. Protein assays were performed on the solubized cell pellets by formation of the cuprous bicinchonicic acid complex using a spectrophotometric microtiter plate reader. The method and reagents used are supplied in a kit (Pierce, Rockford, IL) and were performed according to the manufacturers' directions.

Radioactivity. The content of radioactivity in the tissue samples was measured in a gamma well counter and compared to dilutions of standards.

Statistics. All in vitro experiments were repeated a minimum of six times, each with a minimum of triplicate internal samples for each experiment. Experiments involving tumor-bearing mice were repeated with nine animals each. The tests of statistical function (mean, s.e.m., and unpaired Student's t-tests assuming unequal variances) were performed using Microsoft Excel software.

Characterization of the Phenotype of Transfected Cells

Expression of TfR Protein. Confirmation of expression of TfR protein in the TfR+ cells, and absence in the TfR- cells, was established by Western Blot analysis. Cultured cells were harvested by scraping. SDS-lysates of equal protein content from whole TFR+ and TfR- cells were subjected to SDS PAGE electrophoresis and transferred to PVDF membranes. The primary antibody, HTR0.868.4, used for Western Blot identification of the TfR is a mouse monoclonal directed against the internal (cytoplasmic) tail of the human TfR. This antibody is now marketed as GR09 by Oncogene Research Products/Calbiochem (Cambridge, MA). The secondary antibody (goat antimouse) conjugated to alkaline phosphatase, allowed identification of bands corresponding to the TfR by chemiluminescence using the Western-Light Kit (Tropix, Inc., Bedford, MA). The expected location for the monomeric form of the TfR (expected molecular weight of approximately 90,000) was inferred from the mobility of biotinylated protein molecular weight markers (Tropix, Inc.).

Function of the Transfected TfR: Uptake of Iodine-125-Diferric Transferrin. The functional capacity of the TfR in the transfected cells was established by an assay of accumulation of ^{125}I-Tf by a standard published method (*39*). Monolayers of cultured cells were both first washed, then preincubated at 37° for 2 hr with 5 ml serum-free DMEM to deplete the cells of Tf. The cells were then incubated in HBSS containing 5 n*M* ^{125}I-Tf and 2 mg/ml bovine albumin (BSA) for 30 min at 37°C. In most experiments described here and below, the HBSS is at pH 7.2 and contained 3.7 g/L $NaHC0_3$, 1 m*M* $CaCl_2$, 0.5 m*M* $MgCl_2$ and 0.5 m*M* $MgSO_4$.

Gallium-67 Uptake In Vitro

Tf-Independent Uptake of Gallium-67. Cells were grown in 25-cm (*2*) flasks to a similar degree of subconfluence. Monolayers of cells were both first washed, and then preincubated, at 37° for 2 hr with 5 ml serum-free DMEM to deplete the cells of Tf. The preincubation medium was replaced by 1.5 ml of a solution containing 10 μCi/ml carrier-free ^{67}Ga citrate in HBSS (approximately 0.25 n*M* ^{67}Ga). After incubation at 37° for 30 min, the cells were separated from unbound radioactive contaminating material as described above.

Effect of Calcium on Tf-Independent Uptake of Gallium-67. Cells were incubated with ^{67}Ga-citrate, as described above, except that the concentration of calcium in the HBSS was varied at either 0, 1, 5 or 10 m*M* with $CaCl_2$.

Effect of Iron Salts on the Tf-Independent Uptake of Gallium-67. Cells were incubated with ^{67}Ga-citrate, as described above, except that either 5 or 20 μM iron tricine ascorbate (FeTA) were added both to the serum-free preincubation mixture, as well as to the HBSS incubation mixture. To prepare the FeTA, 45 or 180 μl of 1 m*M* $FeCl_3$ was added to 500 μl of a freshly made solution of 10 m*M* tricine, 0.3 m*M* ascorbate and incubated at room temperature for 30 min. The FeTA mixture was brought to a final volume of 9 ml with either the preincubation or incubation solution for a final concentration of either 5 or 20 μM iron. Controls included cells in which ^{67}Ga was introduced with either HBSS alone, or HBSS plus tricine ascorbate (without iron).

Effect of Purified Apo- and Holo-Tf on Uptake of Gallium-67-Citrate. That TfR+ cells are capable of accumulating ^{67}Ga when it presented as a Ga-Tf complex was confirmed by a method similar to that described above. Before incubation with the cultured monolayers of cells, 67Ga-citrate was first incubated at 37°C for 30 min in HBSS with 2 mg/ml BSA and either 1 or 100 n*M* of purified human apo- or diferric holo-transferrin. The preincubated solutions containing Tf and ^{67}Ga were then added to the monolayers of the cells and the assays performed as above.

Uptake of Gallium-67 by Tumors in Mice

Tumors were grown as explants of TfR+ and TfR- cells in young female nude mice (each weighing approximately 25 gm) of the strain Balb/C Nu/Nu. Cultured cells were harvested by trypsinization, collected and washed once in PBS, and concentrated by low-speed centrifugation. The contents of one confluent 75-cm^2 flask of cells was suspended in a total final volume of approximately 200 μl normal saline, and injected subcutaneously in the left rear flank of each recipient mouse. When tumors had reached a size of approximately 5–8 mm^3, each tumor-bearing mouse was injected intraperitoneally with 50 μCi of carrier-free ^{67}Ga-citrate in a total volume of 150 μl normal saline.

The mice were euthanized by CO_2 inhalation 72 hr after the injection of ^{67}Ga. The mice were dissected and the organs and tissues were removed and weighed. Radioactivity in the harvested tissues was determined by gamma well counting. Only non-necrotic portions of tumors were analyzed.

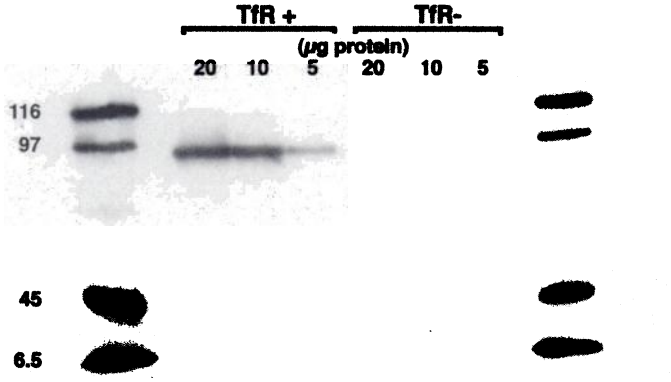

FIGURE 2. Western Blot analysis of protein lysates of TfR+ and TfR- cells. The primary antibody is a murine monoclonal that is directed against the cytoplasmic tail of the human transferrin receptor. In TfR+ cells, but not TfR- cells, the antibody recognizes a protein of the expected molecular weight as the monomeric subunit of the human transferrin receptor (90,000 kD).

RESULTS

Characterization of Phenotype of Transfected Cells

Expression of TfR Protein. Western Blots of TfR+ cells demonstrate a strong band corresponding to the expected mobility of the monomeric human TfR, approximately 90,000 daltons (Fig. 2). The molecular weight of the intact human TfR is 180,000, but in the presence of SDS, dissociation of the TfR dimer occurs. The lysates of TfR- cells contain no protein that reacts with the monoclonal antibody.

Function of the Transfected TfR: Uptake of Iodine-125-Diferric Transferrin. The functional capacity of the TfR was established in TFR+ and TfR- cells by an assay of accumulation of ^{125}I-diferric Tf. When incubated as described above in the presence of diferric Tf, the quantity of Tf accumulated per milligram of total cellular protein is: TfR+ = 7668.18 fmol/mg (s.e.m. 529.20); TfR- cells = 59.89 fm/mg (s.e.m. 9.34). The TfR+ cells accumulate 128-fold more ^{125}I than TfR- cells ($p < 0.001$), confirming that the TfR+ cells, but not the TfR- cells, express a functional TfR in that it is capable of binding and internalizing diferric Tf. The negligible amount of ^{125}I-Tf accumulated by the TfR- cells, only twofold greater than background radioactivity, is likely related to nonspecific binding or pinocytosis.

Gallium-67 Uptake In Vitro

Tf-Independent Uptake of Gallium-67. When Tf is eliminated from the system, there is no significant difference in uptake of ^{67}Ga citrate between TfR+ and TfR- cells (Figs. 3–5). Both accumulate easily measurable amounts of ^{67}Ga in vitro. The overexpression of the TfR in the TfR+ cells, and absence of expression in the TfR- cells, does not affect the Tf-independent uptake of ^{67}Ga.

Effect of Calcium on Tf-Independent Uptake of Gallium-67. Uptake of ^{67}Ga by the Tf-independent route is augmented by increasing the calcium concentration in the incubation solution (Fig. 3). No significant difference in uptake occurs between TfR+ and TfR- cells for any concentration of calcium examined ($p \geq 0.2$). There is no effect in basal level of uptake of ^{67}Ga in either cell line when the amount of calcium in solution is increased from 0–1 m*M* ($p > 0.5$). However, uptake of ^{67}Ga by both cell types increases tenfold over basal levels when the concentration of calcium is increased to 5 m*M* ($p \leq 0.003$), and 75-fold over basal levels when the calcium is increased to 10 m*M* ($p \leq 0.003$).

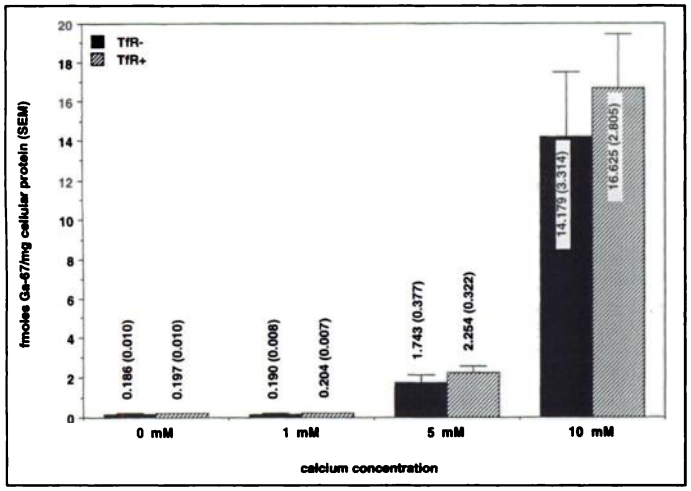

FIGURE 3. Effect of calcium concentration on the Tf-independent uptake of ^{67}Ga by TfR- and TfR+ cells. The TfR+ and TfR- cells were incubated with ^{67}Ga in solutions containing 0, 1, 5 and 10 m*M* $CaCl_2$ for 15 min at 37°C. There is no significant difference in uptake of ^{67}Ga between 0 and 1 m*M* Ca++. At 5 and 10 m*M* Ca++, a significant difference in uptake of ^{67}Ga from basal levels is observed (n = 6).

Effect of Iron on the Tf-Independent Uptake of Gallium-67. There is no significant difference between TfR+ and TfR- cells in uptake of ^{67}Ga for any of the concentrations of FeTA examined ($p > 0.7$) (Fig.4). When both types of cells are preincubated, and then incubated, with ^{67}Ga-citrate in the presence of iron salts, uptake of ^{67}Ga by the Tf-independent process is significantly increased. Incubation of the cells with tricine ascorbate (TA) only (without iron) has no effect on uptake of ^{67}Ga ($p > 0.2$). When experiments are conducted in the presence of 5 μM FeTA, uptake of ^{67}Ga is tenfold greater than when cells are incubated with TA buffer alone ($p < 0.001$). Uptake of ^{67}Ga with 20 μM FeTA is 40-fold greater than that with TA alone ($p \leq 0.0001$).

Effect of Purified Apo- and Holo-Tf on Uptake of Gallium-67. When 0.25 n*M* ^{67}Ga is preincubated with 1 n*M* apo-Tf, TfR+ cells accumulate 2.3-fold more ^{67}Ga than TfR- cells, a difference of statistical significance ($p = 0.03$) (Fig. 5). With 100 n*M* apo-Tf, the TfR+ cells accumulate 24-fold more ^{67}Ga than the TfR- cells, ($p =$

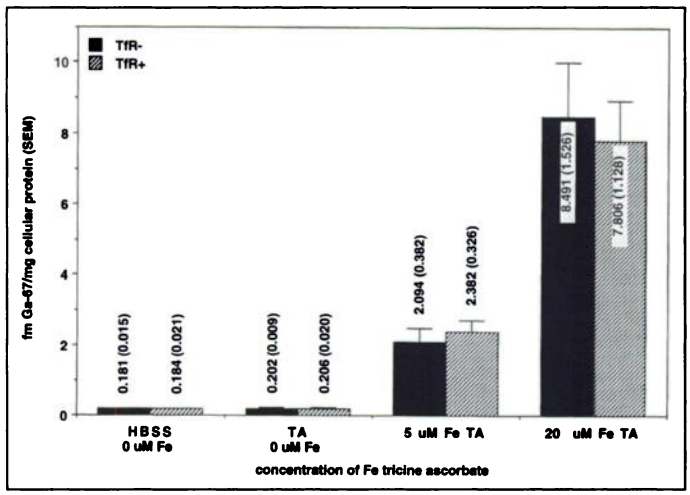

FIGURE 4. Effect of soluble iron concentration on the Tf-independent uptake of ^{67}Ga by TfR- and TfR+ cells. The TfR+ and TfR- cells were first preincubated, and then incubated, with ^{67}Ga in Hank's Balanced Salt Solution (HBSS) containing either 5 or 20 μM iron tricine ascorbate (FeTA), or in TA alone, for 15 min at 37°C. No significant difference in uptake of ^{67}Ga occurs over basal levels (HBSS) when cells are incubated with TA buffer alone. At 5 μM and 20 μM FeTA, a significant difference in uptake of ^{67}Ga from basal levels is observed (n = 9).

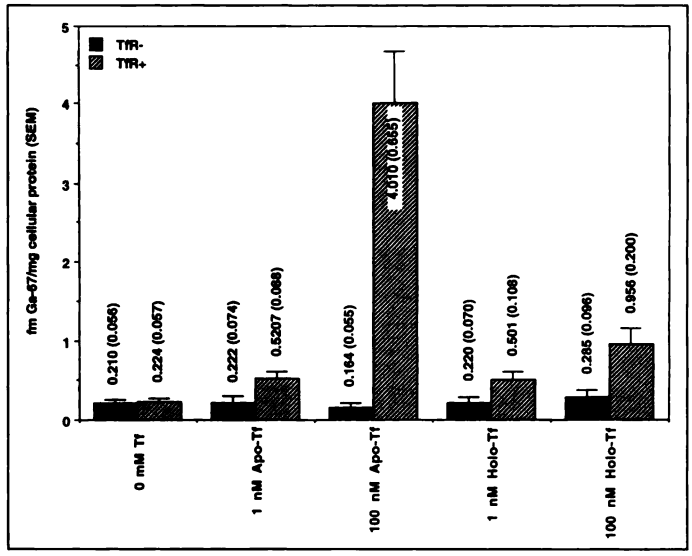

FIGURE 5. Effect of preincubating ^{67}Ga with either apo-transferrin or holo-transferrin. Gallium-67-citrate was preincubated with purified human apo- or holo-transferrin before incubating the ^{67}Ga with cells. TfR+ cells accumulate 2.3-fold more ^{67}Ga in the presence of apo-transferrin than TfR- cells. With 100 n*M* apo-Tf, the TfR+ cells accumulated 24-fold more ^{67}Ga than the TfR- cells. When the ^{67}Ga is preincubated with holo-transferrin, less difference in uptake of ^{67}Ga between TfR+ and TfR- cells is observed. Concentrations of apo- or holo-Tf greater than 100 n*M* did not result in a significant further increase in uptake of ^{67}Ga (data not shown) (n = 6).

0.002). When ^{67}Ga is incubated with holo-Tf before incubation with the cells, there is less difference in uptake of ^{67}Ga between the TfR+ and TfR- cells. For 1 n*M* holo-Tf, although the difference is 2.3-fold greater for uptake of ^{67}Ga by TfR+ cells than the TfR+ cells, this value is not of statistical significance ($p = 0.06$). For 100 n*M* holo-Tf, the 2.7-fold difference between in TfR+ and TfR- is significant ($p = 0.02$). The presence of Tf, either apo- or holo-, does not alter the Tf-independent uptake of ^{67}Ga by TfR- cells ($p > 0.5$). Concentrations of apo- or holo-Tf greater than 100 n*M* do not result in a significant further increase in uptake of ^{67}Ga (data not shown).

Uptake of Gallium-67 by Tumors in Mice

The degree of tumorigenicity and rate of growth of explants of the TfR+ and TfR- cells as tumors in mice are indistinguishable. Both types of tumors grow to 8–10 mm (*3*) in approximately 2.5–3 wk. The degree of necrosis present in the tumors is similar (and small in both). None of the tumors have been found at necropsy to have metastasized distantly. For these experiments, ^{67}Ga was given intraperitoneally, a route that results in a pattern of uptake that we have found to be equivalent to intravenous administration. The ratio of accumulation of ^{67}Ga (per milligram of tissue) in various tissues and organs, relative to that in an equal weight of blood, is shown in Figure 6 for female nude mice bearing tumors grown as explants of either TfR+ or TfR- cells. Between mice bearing TfR+ and TfR- tumors, there is no significant difference in total body retention of ^{67}Ga 72 hr postinjection of the radionuclide (data not shown), or in the tissue-to-blood ratios of ^{67}Ga accumulation for any of the normal tissues and organs examined ($p > 0.2$). The TfR+ tumors accumulate tenfold more ^{67}Ga than TfR- tumors ($p < 0.001$).

DISCUSSION

The pair of transfected cells described in this report represent a controlled system for studying the relative importance, both in vivo and in vitro, of both Tf-dependent and Tf-independent systems for the uptake of ^{67}Ga. These cells will be used for

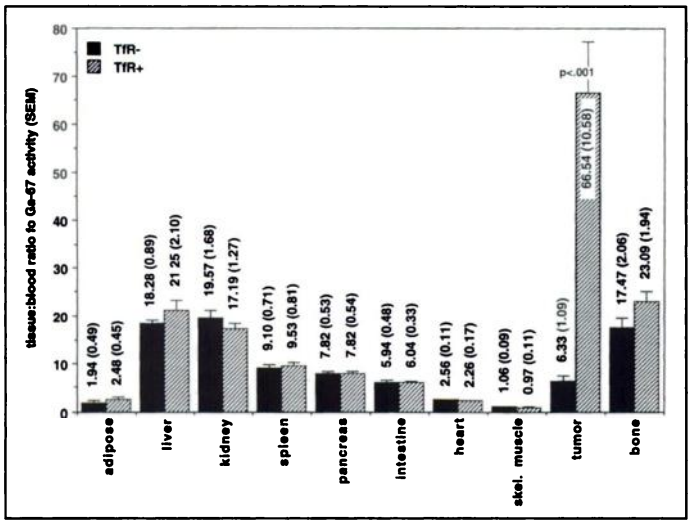

FIGURE 6. Uptake of ^{67}Ga in nude mice bearing TfR+ or TfR- tumors. Tumors were grown as subcutaneous explants of TfR+ and TfR- cells in young female nude mice. When tumors reached 5–8 mm^3 diameter, mice were injected intraperitoneally with 50 μCi of carrier-free ^{67}Ga-citrate. At 72 hr, the mice were killed and dissected. The ratios of uptake of ^{67}Ga for various tissues and organs, relative to an equal weight of blood, are shown. Between mice bearing TfR+ and TfR- tumors, there was no significant difference in the tissue-to-blood ratios of ^{67}Ga accumulation for any of the normal tissues or organs examined. The TfR+ tumors are intensely gallium avid, accumulating tenfold greater amounts of ^{67}Ga than TfR- tumors ($p < 0.001$; $n = 9$).

future studies aimed at manipulating the two systems. They also may prove useful in the study of iron physiology. The cells offer an alternative to conventional systems in that they are identical except that one cell line (TfR-) has no TfR. In the other (TfR+) the human TfR is over-expressed in a manner independent of cell growth or iron content, which could alter the cells metabolically in many ways that may confound a well-controlled experimental determination of cause and effect. We have confirmed the phenotype of these transfected cells. By Western Blot analysis, only the TfR+ cells contain a protein of the expected mobility for the monomeric TfR (90,000 kD) which reacts with a monoclonal antibody directed against the cytoplasmic tail of the human TfR. The TfR synthesized by the TfR+ cells is functional in that it will bind and internalize [^{125}I]-diferric Tf.

When Tf is eliminated from the incubation solution, there is no significant difference in uptake of ^{67}Ga-citrate between TfR+ and TfR- cells. This suggests that the degree of expression of the TfR does not affect uptake of ^{67}Ga by the Tf-independent pathway.

TfR+ cells accumulate greater quantities of ^{67}Ga than TfR- cells when the ^{67}Ga is presented as a Ga-Tf complex in a manner that is concentration dependent and saturable. No attempt was made to separate, either by dialysis or column filtration, the unbound ^{67}Ga from that bound to Tf. Our experience agrees with that published by others that ^{67}Ga elutes very easily from Tf and, even at neutral pH in normal saline, ^{67}Ga can be continually dialyzed or easily separated from Tf (*4,13–15*). Instead, we chose to use molar concentrations of apo-Tf that greatly exceed those of ^{67}Ga, to favor formation of the Ga-Tf complex. When the ^{67}Ga was preincubated with holo-Tf, which is highly iron-saturated, there is less difference in uptake of ^{67}Ga between the TfR+ and TfR- cells. These results are consistent with previous observations that ^{67}Ga cannot displace iron from Tf or binds poorly to monoferric Tf. The presence of Tf, either apo- or holo-, does diminish the Tf-independent uptake of ^{67}Ga by TfR- cells. Either significant ^{67}Ga remains unbound even when an excess of Tf is available, or ^{67}Ga can be easily removed from Tf for uptake by the Tf-independent system.

Uptake of ^{67}Ga by a Tf-independent route is significantly augmented by two in vitro manipulations that also have been shown to promote the uptake of iron by the Tf-independent system (*26*, *40*). These are an increase in the calcium concentration and an increase in the concentration of soluble (free ionic) iron in the incubation solution. Again, no significant difference in uptake of ^{67}Ga occurs between TfR+ and TfR- cells for any concentrations of calcium or iron examined.

Normal mammalian physiological levels of serum calcium range from 2–3 m*M*, with free calcium being 1–2 m*M*. Humans become symptomatic when free serum calcium levels rise above 3 m*M*. In these experiments, which used free calcium in the absence of serum proteins, the concentrations needed to promote the uptake of ^{67}Ga by the Tf-independent route exceed those that would be compatible with normal physiology by two- to threefold. The mechanism by which calcium acts to augment ^{67}Ga and whether a safe in vivo correlate might exist for exploiting the calcium effect to promote the Tf-independent uptake of ^{67}Ga are as yet unknown. Whether a calcium effect could be achieved in the physiologic milieu of serum proteins also is not known.

That ferric chloride stimulates the uptake of ^{67}Ga in a system free of Tf previously has been reported by Chitambar and Sax (*10*). They also have shown the converse: that gallium-nitrate stimulates the uptake of iron nitrilotriacetic acid (FeNTA). Chitambar's data supports his hypothesis that ^{67}Ga shares the same system described by Kaplan et al. (*26*) for the Tf-independent uptake of iron (*8,10*). As a salt of tricine ascorbate, which stimulates uptake of ^{67}Ga in our experiments, iron exists in both the ferric and ferrous form. Whether uptake of ^{67}Ga by the Tf-independent route is stimulated by both forms is not known from our experiments. However, Kaplan's work suggests that both divalent and trivalent iron stimulates uptake of the Tf-independent system for the uptake of iron and several other metals (*26*). Although highly iron-saturated, the presence of holo-Tf in the above experiments does not enhance the uptake of ^{67}Ga by TfR- cells. This suggests that the effect of iron in stimulating the uptake of ^{67}Ga by the Tf-independent pathway must be due to free iron, and not that which is bound to Tf. However, the amount of iron present in the experiments testing the effect of holo-Tf was approximately fivefold less than the amount of iron presented as FeTA.

Our data support the traditional belief that increased TfR density in tumors results in gallium avidity. In nude mice, the TfR+ tumors are intensely gallium avid, compared to all other normal tissues and organs examined, and accumulate tenfold greater amounts of ^{67}Ga than do the TfR- tumors. However, the in vitro levels of uptake of ^{67}Ga that could be induced by the Tf-independent pathway in both TfR+ and TfR- cells actually exceed those that can be achieved in TfR+ cells by the Tf-dependent route. While TfR expression may be sufficient to result in Ga avidity, our previous data in hypotransferrinemic mice suggest that some tumors may be Ga avid because of enhanced uptake by a Tf-independent pathway (*28*). If the same incremental increases in uptake of ^{67}Ga by the Tf-independent pathway can be produced in vivo as are seen in vitro, then tumor imaging with ^{67}Ga might be more broadly applicable to tumors both with Tf-dependent and Tf-independent uptake of ^{67}Ga.

CONCLUSION

The in vivo uptake of ^{67}Ga is significantly increased in tumors in which the TfR is overexpressed, compared to those

without a functional Tfr. However, a notable amount of accumulation of ^{67}Ga also occurs, both in vitro and in vivo, by a Tf-independent route. In vitro experiments demonstrate that the uptake of ^{67}Ga by the Tf-independent route can be regulated and enhanced to levels that equal or exceed those achieved by the Tf-dependent route. These findings suggest that further experiments to manipulate the uptake of ^{67}Ga in vivo to enhance the utility of the radiometal for tumor imaging may be worthwhile.

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