Evaluation of Human Transferrin Radiolabeled with N-Succinimidyl 4-[Fluorine-18](Fluoromethyl) Benzoate

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Iron metabolism plays a key role in cell proliferation and survival in rapidly growing cancer cells. Uptake is mediated by the carrier protein transferrin. The increased need for iron has been used as a method to target tumors and there is well-documented evidence that certain tumors can be imaged with tracers such as ^{67}Ga, that mimic transferrin-mediated iron uptake. To obtain a tracer that would be better able to quantitate transferrin kinetics and indirectly evaluate iron metabolism, we have labeled human transferrin with the positron emitter, ^{18}F, with a one-step high-specific activity method developed in our laboratory. **Methods:** We measured the binding affinities of [^{18}F]diferric (holo-) and iron-free (apo-) transferrin on two human cell lines. We also compared cellular uptake of [^{18}F]holo-transferrin and [^{67}Ga]citrate in various conditions, and washout of label incorporated into cells. **Results:** The binding affinity of [^{18}F]holo-transferrin was found to be the same as that reported for [^{125}I]holo-transferrin. In our hands there was no significant difference in binding affinity between diferric holo-transferrin and iron-free apo-transferrin. [^{18}F]holo-transferrin uptake rapidly reaches a steady-state equilibrium between the intracellular and extracellular environment, while gallium accumulation linearly increases with time. [^{18}F]holo-transferrin is rapidly recycled out of the cell with similar kinetics to those reported for [^{125}I]holo-transferrin. **Conclusion:** [^{18}F]holo-transferrin displays the properties of native transferrin and appears suitable for quantitative evaluation of transferrin kinetics in vivo.

Key Words: transferrin; fluorine-18; gallium-67; neoplasms; iron metabolism

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In our search for biochemical probes that will be early, sensitive indicators of rapidly growing tumors, we are investigating a number of different receptor-binding radiotracers (*1*). Iron metabolism plays a key role in the survival of rapidly proliferating cells, since iron is a cofactor in several metabolic pathways (*2*). Key enzymatic activities, such as ribonucleotide reductase (*3*), and vital energetic pathways, such as the electron transport chain (*4*), rely on iron to function. An efficient transport and uptake system guarantees adequate delivery of this element to tissues.

In the blood, absorbed iron is tightly bound by the transport protein transferrin (Tf). One Tf molecule can bind up to two iron atoms. The Tf-iron complex (diferric or holo-transferrin) is taken up by cells via receptor-mediated endocytosis through the Tf-receptor. Iron is deposited inside cells after release from Tf in an intracellular compartment at low pH (*5,6*). Iron-free (apo-) Tf is then rapidly recycled back to the cell surface and out of the cell where it can bind to newly available iron. Since no iron radioisotope is suitable for imaging, one can indirectly measure iron uptake by quantitating Tf uptake. Many tumors appear to have not only high iron requirements, but also express more Tf-receptors on the cell surface (*7,8*).

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The iron requirement of certain cell types has long been investigated as a way of targeting cancer cells. For example, ^{67}Ga has been used for tumor imaging for many years. It is generally accepted that gallium mimics iron in its ability to bind to transferrin, and its uptake into cells is transferrin-mediated. However, Larson et al. (*9,10*) and, more recently, Harris et al. (*11*) report a low affinity constant for [^{67}Ga]Tf. Work by Brunetti et al. (*12*) showed that the formation of Ga-Tf in vivo cannot be accurately measured in vitro. There is also some evidence that in certain situations gallium may be taken up by cells in a non-transferrin dependent manner (*13*). Furthermore, formation of such a complex is dependent on the concentration of other compounds such as bicarbonate (*14*), so differences in blood bicarbonate concentrations between individuals will also influence how much complex is formed. Other means of labeling transferrin such as the use of ^{123}I or ^{111}In have limitations which will be discussed.

To develop a positron-emitting radiotracer for use with PET that would be able to quantitate Tf uptake in vivo, and to obtain a more sensitive method to detect cancer cells based on Tf-receptor expression, we have investigated the properties of Tf covalently labeled with ^{18}F at high specific activity using a one-step method (*15*). Some of the data we report have already been presented in preliminary form (*16*).

MATERIALS AND METHODS

Cell Culture

Cells were grown in a humidified atmosphere containing 5% CO_2 and 95% air at 37°C. For binding and uptake experiments we used U937 cells, a human hystiocytic lymphoma cell line that is known to express large amounts of Tf-receptor (*17,18*). Cells were grown at a density of 2–5 × 10^5 cells/ml in complete medium (RPMI-1640 supplemented with 10% fetal bovine serum [FBS]). Binding experiments were also performed on A431 cells, a human epidermoid cancer cell line originally obtained from G. Todoaro (NIH). Cells were grown in complete medium, the same as above, until confluent (approximately 1 wk) and passaged (1 to 4–1 to 6) after trypsinization.

Radiolabeling

Iodine-125-holo-transferrin (specific activity = 115 Ci/mmole) and human apo- and holo-transferrin were obtained commercially. The proteins were dissolved in 0.1 *M* borate buffer (pH 8.5) to a final concentration of 1 μg/μl. We added 100 μl of these solutions to a 1.5-ml Eppendorf tube containing 2–4 mCi of N-succinimidyl 4-[^{18}F] (fluoromethyl) benzoate (*15*). The mixture was allowed to react for 10 min at room temperature after vortexing. Size-exclusion HPLC was then performed using phosphate buffered saline (PBS; pH 7.4) as the eluant. The fraction containing the labeled protein was collected, and the radiochemical purity was determined by silica gel thin-layer chromatography. The specific activity was between 1000 and 2000 Ci/mmole (end of synthesis) and the radiochemical purity more than 99%.

Binding Assays

For binding assays with [^{18}F]holo-transferrin on U937 cells at 4°C, 50 ml of cell suspension were collected and washed twice with 50 ml ice-cold RPMI-1640 with 0.1% bovine serum albumin (BSA). For binding experiments using [^{18}F]apo-transferrin, 25 μM deferoxamine mesylate (Sigma Chemical Corp., St. Louis, MO) was also added to chelate the iron present in the medium. Alternatively, binding assays were performed on cells in which ATP formation, and consequently receptor internalization, had been blocked by washing twice and then incubating for 1 hr with 15 ml of RPMI-1640, supplemented with 0.1% BSA and 0.02% sodium azide at 37°C. For all binding assays, cells were resuspended at a concentration of 0.5–1 × 10^7/ml in 500-μl tubes and incubated under continuous agitation for 90 min at 4°C or 37°C, with increasing concentrations (range ~10 pM to ~1 μM) of [^{18}F]apo- or holo-transferrin. Bound radioactivity was separated by centrifuging the cells through dibutyl phthalate as previously described (*5*) and measured with a gamma counter. Triplicate measurements were obtained for every concentration point. Affinity constants and receptor concentrations were calculated by Scatchard analysis using a dedicated computer program (*19*). Control experiments were performed under the same conditions using unlabeled holo- and apo-transferrin (range ~10 pM to ~1 μM) to displace [^{125}I]holo-transferrin.

Binding assays using A431 cells were carried out in six-well plates at 4°C. Cells were plated at approximately 2 × 10^6/well. The assay was performed on the next day (confluent cells). The same procedure was used as described for U937 cells, with the exception of the harvesting of cell-bound radioactivity. After 90 min of incubation at 4°C under continuous agitation, cells were washed three times with 5 ml RPMI-1640 + 0.1% BSA, and the bound counts were recovered by solubilizing the cells with three washes with 0.1 N NaOH (5 ml/wash) and collecting all the resulting liquid in a scintillation vial.

In separate experiments we determined the relative binding of [^{18}F]holo-transferrin versus native holo-transferrin on A431 cells. Affinity constants and receptor concentrations were determined both by Scatchard analysis and by displacing tracer amounts of [^{18}F]holo-transferrin with the cold protein taken from the same lot used for labeling.

[^{18}F] and [^{125}I] Holo-Transferrin Uptake and Washout in Viable Cells

The U937 cells were washed twice and incubated for 1 hr at 37°C in RPMI-1640 + 0.5% tissue culture grade BSA. Approximately 1 × 10^7 cells were resuspended in the same medium at a concentration of 2 × 10^5 cells/ml in a tissue culture flask. After adding tracer amounts (~10 pM) of [^{18}F] holo-transferrin or [^{125}I] holo-transferrin, we placed the cells in a 37°C incubator and collected duplicate 1-ml samples from the cell suspension at different time intervals between 0 and 150 min after adding the label. Cell-bound radioactivity was determined in the same fashion as for the binding assays.

To determine the rate of Tf washout, U937 cells were prepared in parallel with the uptake experiments. Cells were allowed to incorporate the labeled Tf for 60 min at 37°C. Cells were washed twice with ice-cold, protein-free RPMI-1640, and the surface-bound Tf was thereafter stripped by incubating for 30 min in RPMI-1640 + 1 mg/ml of the nonspecific protease pronase (*20*). After being washed twice in ice-cold RPMI-1640 + 0.5% tissue culture grade BSA, the cells were placed in preheated RPMI-1640 + 10% FBS at 37°C and replaced in the incubator. Cell-associated radioactivity and counts in the medium were then determined serially at 0, 10, 20, 30, 45 and 60 min. To prove that the label released back to the medium was still intact [^{18}F]Tf, we performed size-exclusion HPLC and SDS polyacrylamide gel electrophoresis using the cell supernatants collected during the washout period. Immunoprecipitation using an anti-Tf antibody-protein-A resin conjugate was also performed on these samples. This technique is described in the section on gallium-Tf complex formation.

Time-activity curves were analyzed using a Macintosh computer and Kaleidagraph software (Abelbeck Software, Version 3.0.4, distributed by Synergy Software, Reading, PA) for linear and exponential fits of data points or B-opt and P-opt, a compartmental analysis program written by Michael M. Graham, University of Washington Medical Center, Seattle, WA (*21*).

Gallium-67 Uptake and Washout

Carrier-free ^{67}Ga-citrate was obtained commercially. Cells were washed twice with RPMI-1640 + 0.5% BSA at the beginning of the experiment. The ability of U937 cells to take up gallium was tested under four different conditions:

1. 2 × 10^5 cells/ml were incubated at 37°C with a freshly prepared transferrin-free solution of RPMI-1640 + 0.5% BSA + 1 μCi/ml ^{67}Ga-citrate as supplied by the manufacturer.
2. 2 × 10^5 cells/ml were incubated at 37°C in RPMI-1640 + 0.5% BSA + 10% of a 20 μM (approximate concentration present in serum) apo-Tf solution in protein-free RPMI-1640, previously incubated for 30 min with 10 μCi/ml ^{67}Ga-citrate.
3. (c) 2 × 10^5 cells/ml were incubated at 37°C in RPMI-1640 + 10% FBS previously incubated for 30 min at 37°C with 10 μCi/ml ^{67}Ga-citrate.
4. 2 × 10^5 cells/ml were incubated at 37°C in RPMI-1640 + 10% rat serum (Sprague-Dawley; 280 g obtained 30 min after intravenous injection of 500 μCi ^{67}Ga-citrate.

As for the previously described uptake determinations, duplicate 1-ml samples were collected at different time points for 5 hr, and the cell-associated radioactivity was measured. After allowing 12 hr for gallium uptake, washout of the label from cells was evaluated as described in the previous section.

Determination of Gallium-67-Transferrin Complex Formation

Gallium-Tf formation was evaluated by immunoprecipitating the Tf from the solutions used for the ^{67}Ga uptake experiments using a rabbit antiserum against human Tf and measuring the antibody-bound radioactivity. Ten milligrams of protein-A agarose beads were incubated for 2 hr at room temperature with a 1:1000 dilution of the Tf antiserum in 1 ml of RPMI-1640 + 0.1% BSA in 1.5-ml tubes at room temperature while rotating. Excess antibody was removed by washing the resin twice with 1 ml of the same solution free of antibody. Aliquots of the four ^{67}Ga solutions (~100,000 c.p.m each) described in the previous section were added to the beads and incubated for 6 hr at room temperature. The tubes were centrifuged, and the resin-bound and free radioactivity were determined. Fluorine-18-Tf and ^{125}I-Tf binding to the beads was determined in similar fashion as a control.

RESULTS

K_d and B_{max} for Holo- and Apo- Fluorine-18-Transferrin

Scatchard analysis was performed at 4°C on U937 cells using holo- and apo-[^{18}F]transferrin as ligands. The K_d for holo-Tf was found to be 1.9 ± 0.8 nM (mean ± s.d.). Despite previous reports (*5,6*) of low or no binding of apo-Tf to the Tf-receptor, our experiments performed in the presence of the chelating agent deferoxamine mesylate, in order to block holo-Tf formation in the samples, show a K_d of 4.2 ± 1.7 nM (mean ± s.d.). The apparent number of receptors per cell, although somewhat variable, was similar for the two ligands (1.2 ± 1 × 10^5 sites/cell for holo-Tf; 0.9 ± 0.5 × 10^5 sites/cell for apo-Tf). We

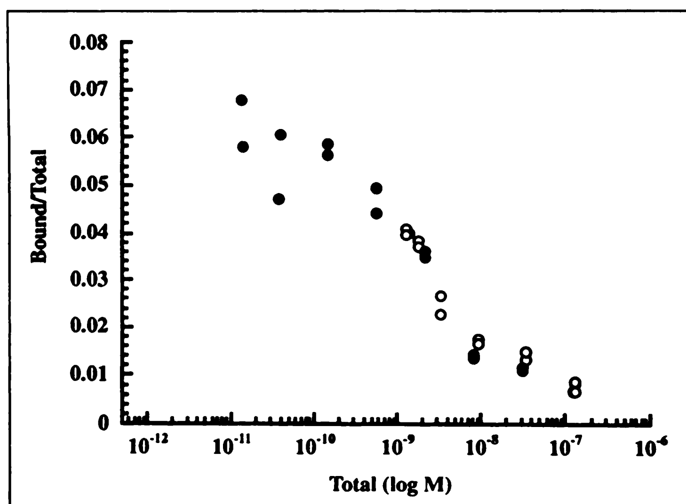

FIGURE 1. Relative binding of [^{18}F]holo-transferrin to A431 cells. Cells were incubated with different concentrations of labeled only (closed circles) or labeled and unlabeled (open circles) [^{18}F]holo-Tf.

also determined the binding affinity of [^{18}F]holo-Tf for the receptor at 37°C. In order to stop Tf internalization at this temperature, cells were treated with sodium azide. At this temperature the K_d was estimated to be 9.2 ± 9.6 n*M* and the number of available receptors was 3.4 ± 0.8 × 10^5 per cell.

K_d and B_{max} for [^{18}F]holo-Tf were also determined in A431 cells at 4°C by Scatchard analysis. The K_d for the Tf-receptor of our labeled protein was measured at 5.2 ± 3.6 n*M*, very close to the value found in U937 cells. The number of sites per cell was 6.5 ± 3.1 × 10^4. These values are the average of three separate experiments.

Relative Binding of Fluorine-18-Holo-Tf

We performed parallel determinations by Scatchard analysis and displacement of holo-Tf on A431 cells. Figure 1 shows results of this experiment. The amount of protein binding to cells at different concentrations does not change if we use labeled Tf alone or unlabeled Tf displacing tracer amounts of ^{18}F-labeled protein. This implies that the labeling procedure does not impair the ability of the protein to recognize cell surface receptors.

Gallium-Tf Complex Formation

Immunoprecipitation of the four gallium solutions described in the methods section showed poor formation of ^{67}Ga-Tf, with <5% of ^{67}Ga partitioning with the anti-transferrin antibody-bound resin. In contrast, more than 90% of the control ^{125}I- and ^{18}F-labeled transferrin bound the antibody conjugated resin.

Uptake and Efflux of Tf and Gallium from Cells

The ability to accumulate [^{18}F]holo-Tf and [^{125}I]holo-Tf was tested in U937 cells. Results are shown in Figure 2. Uptake of both tracers rapidly increased in the initial 15 to 30 min. This was followed by a plateau in the cell-bound radioactivity. On the other hand, all gallium solutions used show linear increase of uptake with time as shown in Figure 3. Of the four solutions, the one obtained by directly injecting ^{67}Ga-citrate in a living rat yielded the highest percentage of uptake (Solution 4).

To establish the fate of [^{18}F]holo-Tf once inside the cell, we evaluated the efflux of radiolabeled Tf from the cells (Fig. 4). The cells that incorporated both ^{18}F and [^{125}I]holo-Tf, rapidly released it during the 37°C incubation, with a half-life of approximately 10 min. Under similar conditions there was no release of gallium from cells following its uptake from medium containing 10% rat serum (Solution 4; see Materials and Methods; Fig. 4).

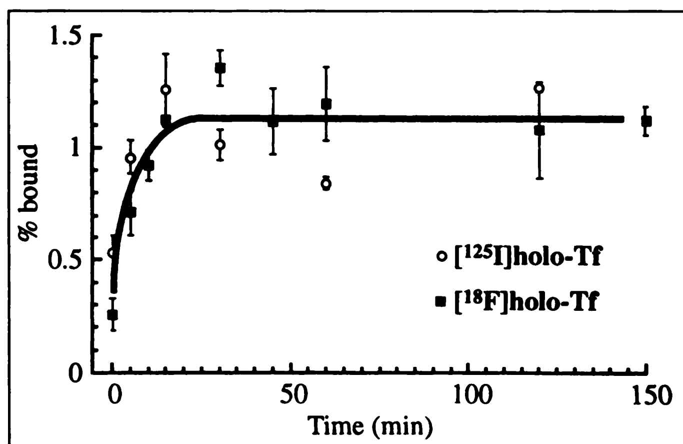

FIGURE 2. Uptake of tracer amounts of ^{125}I and [^{18}F]holo-transferrin by U937 cells in serum-free medium (error bars = s.d.; see Materials and Methods).

HPLC and SDS-polyacrylamide gel electrophoresis were performed on the labeled material released from the cells incubated with [^{18}F]holo-Tf; these experiments showed elution patterns and migration identical to intact transferrin (Fig. 5). Furthermore, more than 90% of the protein released into the culture medium was immunoprecipitated with the anti-transferrin antibody.

DISCUSSION

The physiologic role of transferrin is to shuttle iron inside cells, return to the extracellular environment, bind more iron and repeat the process. This is one of the few ligands that survives enzymatic degradation inside the cell and after traveling inside the cell is released to the extracellular environment intact. The principal mechanism by which transferrin escapes such degradation is that after internalization the protein remains bound to the Tf-receptor. After internalization through receptor-mediated endocytosis, coated vesicles go to an intracellular compartment at a pH low enough to allow the release of iron from the protein. Iron-free apo-transferrin maintains high affinity for the Tf-receptor in these conditions and thus follows the pathway of the vesicles back to the cell surface. When the apo-Tf-Tf-receptor complex is again exposed to physiologic pH on the external surface of the cell membrane, the protein is released from the receptor. The explanation for this phenome-

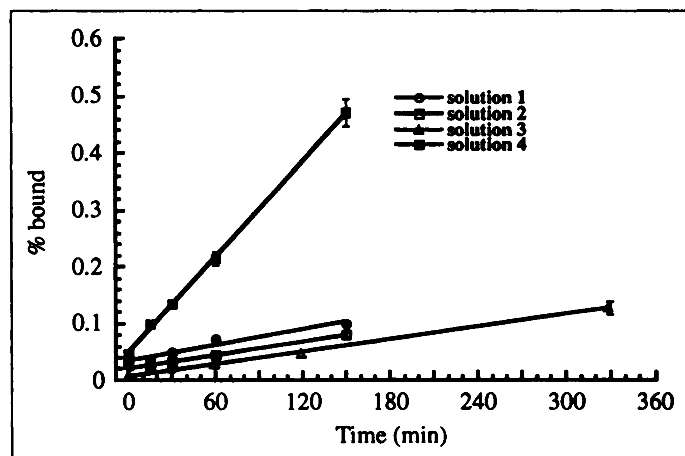

FIGURE 3. Gallium-67 uptake in U937 cells. The cells were incubated for different times with four different ^{67}Ga-containing solutions (error bars = s.d.; see Methods). Solution 4 obtained by injecting [^{67}Ga] citrate in a living rat and recovering its serum after 30 min, consistently yielded higher uptake than the other solutions.

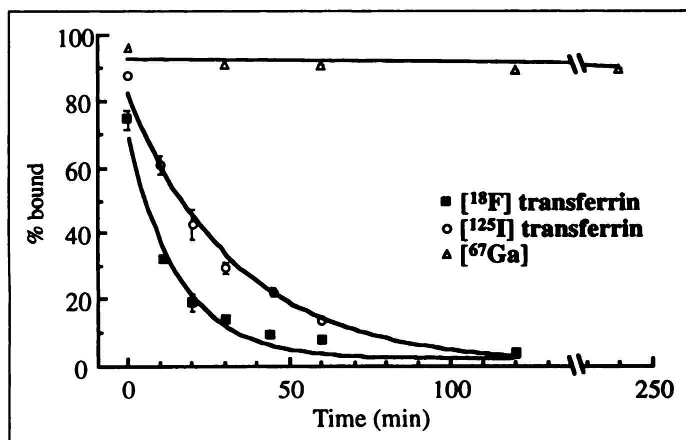

FIGURE 4. Efflux of cell-bound radiolabeled Tf from U937 cells (error bars = s.d.). Cells were incubated for 1 hr with [^{125}I] or [^{18}F]holo-transferrin and for 12 hr with gallium Solution 4 (see Methods). Surface-bound transferrin was then cleaved by incubation at 4°C for 30 min with the protease pronase. Cells were placed back in culture and cell-bound radioactivity was measured at different time points.

non is that apo-Tf has a much lower affinity for the Tf-receptor at pH 7.4 (*22*).

The binding assays were performed at 4°C because evidence suggests that at this temperature there appears to be no internalization of the Tf-Tf receptor complex, and binding properties of the protein for its receptor can be accurately assessed (*5*). The U937 cell line was chosen because these cells express many Tf receptors on their surface and also because these cells grow in suspension, giving the opportunity to separate activity bound to cells by simple centrifugation (*17*). The values we measured for [^{18}F]holo-Tf at this temperature were very close to those reported by others (*5,6*). This parameter, therefore, is not altered by labeling with our compound.

Measurement of K_d and B_{max} was also performed at 37°C on these cells to assess the effect of temperature on receptor binding by [^{18}F]holo-Tf. Receptor internalization was blocked by incubating cells in the presence of sodium azide. It has been shown that treating cells with this compound produces a decrease in the rate of internalization of the Tf-Tf-receptor complex similar to the decrease achieved by incubating the cells at 4°C (*5*). The calculated affinity constants did not differ much from those measured at 4°C, but there was a slight increase in the apparent receptor number (see Results).

We were expecting to find different binding affinities for holo- and apo-Tf, as previously shown. However, there is a large degree of variability in the values reported. Dautry-Varsat et al. (*6*) reported that apo-Tf shows no surface binding at physiological pH on Hep-G2 cells. Klausner et al. (*5*) reported a 20-fold lower affinity (~50 n*M*) for the receptor of apo-Tf compared to holo-Tf on K562 cells. We planned to take advantage of the low affinity of the iron-free form to accurately measure nonspecific binding to the receptor. However, the data we obtained did not show significant differences in K_d between the two protein forms (2 versus 4 n*M*). Subtle differences in the experimental procedures we used may have caused the discrepancy between our data and the previously published work, although every effort was made to avoid or remove iron from our samples. As a result, all further experiments were done using [^{18}F]holo-Tf alone.

Fluorine-18-labeled Tf shows the same immunoreactivity as ^{125}I-Tf. The biologic activity of the protein also appears to be untainted using this labeling procedure. Given the high molecular weight of Tf (~79 kDa) and the low molecular weight of the labeling compound, the addition of the latter to the molecule should not alter protein conformation. Furthermore, on such a large molecule, the high number of potential labeling sites gives a spectrum of proteins labeled on different lysine residues. It is conceivable that addition of the labeling compound to certain eligible residues may alter some properties of the molecule, but these species would only account for a small percentage, if any, of the total labeled protein.

It has been reported that the complex formed between Tf and gallium is rather unstable (*12*). Measurement of gallium-Tf complex formation is not as straightforward as measuring iron-Tf formation (*23*). We were not able to measure binding of gallium to transferrin with our immunoprecipitation assay. The explanation may be that diluting the gallium solutions when incubating with the antibody favors a fast release of gallium from the Tf, given the low affinity of the gallium-Tf complex.

The ^{67}Ga uptake values were rather low (Fig. 3) but are in the same range as those reported in previous work that used a similar model (*24*). Higher uptake values were measured for U937 cells incubated with ^{67}Ga-labeled rat serum (Solution 4). The other three solutions used showed similar uptake patterns, with lower absolute values. The explanation may be that labeling of Tf in vivo is more efficient than the other methods we tested, or that unknown mechanisms favor the cellular uptake of gallium that has been injected into a living animal.

The biological behavior of Tf labeled according to our scheme appears to be identical to that reported for ^{125}I-Tf. Fluorine-18-holo-Tf uptake increases linearly with time and then reaches a steady state between cell-bound and free radioactivity. These findings are consistent with the cycling kinetics of the protein inside the cell. The low uptake values for [^{18}F]holo-Tf were most likely due to the dilute cell concentrations we used to maintain high Tf-receptor expression. These values, however, are very similar to those found by Klausner et al. who used K562 cells to study transferrin kinetics (*5*). Our experiments, aimed at determining the fate of the protein once inside the cell, show that [^{18}F]Tf indeed is rapidly exocytosed after internalization. Furthermore, we have determined that molecular weight and immunoreactivity of the label released from cells is consistent with the protein not being degraded during the cycling process. This has important implications for the potential use of this protein as an imaging agent. Given the

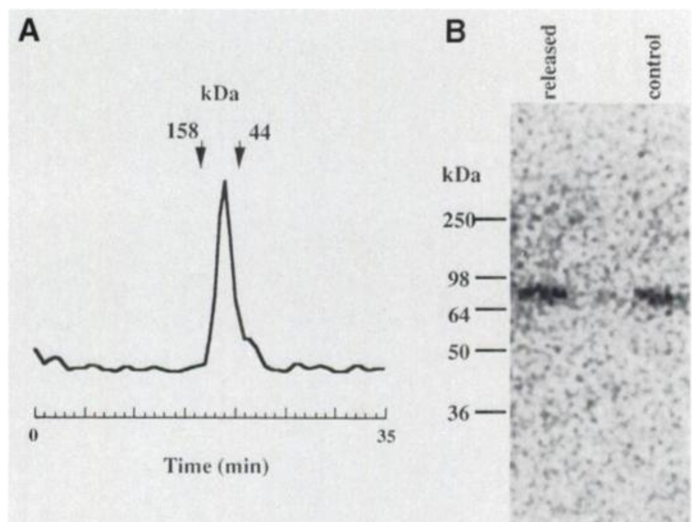

FIGURE 5. (A) HPLC elution pattern and (B) SDS-polyacrylamide electrophoresis on label released from U937 cells. Cells were incubated for 1 hr with [^{18}F]Tf (see Methods), treated with pronase at 4°C for 30 min and then placed back in culture for 30 min. Aliquots of the label released from the cells were analyzed with the two techniques. Elution pattern and migration show a protein with a molecular weight of approximately 80 kDa.

cycling nature of Tf and the fact that our labeling compound is not removed from the protein during this process, it follows that after completing one cycle inside the cell, [^{18}F]Tf can potentially bind more Tf-receptor sites, which would be important in an in vivo situation for maintaining target to nontarget ratios.

There are other possible ways of conveniently radiolabeling transferrin, such as forming a [^{111}In]Tf complex or covalent labeling with ^{123}I. These methods only allow for single-photon imaging. Furthermore, the [^{111}In]Tf complex, although having a much higher affinity constant as compared to [^{67}Ga]Tf, appears to have a lower affinity for the Tf-receptor (*23*). The development of a positron emitting ligand that faithfully reproduces transferrin turnover should offer a great advantage for applications in clinical oncology. The superior sensitivity and resolution of the PET imaging approach make it a more valuable tool compared to single-photon techniques in localizing disease throughout the body (*25*).

CONCLUSION

The ability to accurately measure the rate of transferrin internalization and turnover may yield important information on tumor biology and a new probe for localizing disease. Our data show that labeling transferrin with ^{18}F according to our scheme does not impair the biological properties of the protein and that the behavior of ^{18}F-labeled transferrin in our in vitro experiments is very similar to that of native transferrin. Future studies will assess the potential of [^{18}F]holo-transferrin as a tumor-localizing agent in animal models.

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