A Transferrin-Mediated Uptake of Gallium-67 by EMT-6 Sarcoma. I. Studies in Tissue Culture

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We have studied the in vitro uptake of gallium-67 by exponentially growing EMT-6 sarcoma cells in long-term tissue culture. In this system, the addition of transferrin to the medium was required before an appreciable cellular uptake of Ga-67 occurred. The transferrin effect was complex, with an initial stimulation to a peak cell-to-medium ratio of 8–10:1 at low concentrations of transferrin (0.2 mg/ml), followed by a gradual decline in uptake as transferrin in the medium was increased further. EMT-6 tumor-cell uptake of Ga-67 was probably mediated by a specific cellular receptor for transferrin. Scatchard analysis of the EMT-6 cellular binding of human transferrin labeled with iodine-125 indicated a cellular receptor with affinity for transferrin of 5×10^6 l/mole and abundance of 500,000 receptors per cell. Over the experimental range of transferrin concentration in the medium, the observed uptake of Ga-67 was closely correlated with the degree of formation of Ga-67-labeled transferrin and the fraction of transferrin bound to the cellular receptor (N = 69, r = 0.86, p < 0.0001).

Nearly a decade has passed since Hayes and Edwards observed that carrier-free gallium-67 citrate localized in tumors (*1,2*). Subsequently, Ga-67 has become a broadly applied clinical tool for the imaging of a variety of epithelial and lymphoreticular neoplasms (*3–10*). To date, however, the mechanism whereby Ga-67 is localized in tumors has not been definitely established.

In order to understand better the mechanism of Ga-67 uptake by tumors, we studied the mouse sarcoma EMT-6. This tumor grows both in vitro as a tissue monolayer in long-term tissue culture and in vivo as a transplantable, solid, subcutaneous tumor of BALB/c mice (*11*). In this paper we report

the results of in vitro studies of Ga-67 uptake by EMT-6.

Several authors have studied the uptake of Ga-67 by tumor cells growing in vitro (*12–14*). A particularly interesting aspect of this work is the effect of transferrin on the in-vitro uptake. The results are conflicting. Sephton and Harris (*12,13*) reported enhancement of Ga-67 uptake by transferrin, whereas Gams and associates (*14*) reported inhibition. We found that there was a transferrin-enhanced uptake of Ga-67 into EMT-6, apparently mediated by a transferrin-specific cellular receptor.

MATERIALS AND METHODS

EMT-6 cells were routinely grown in vitro by seeding 2.5×10^5 EMT-6 tumor cells per small Falcon tissue-culture bottle (25 cm² growth area) in 5 ml of Waymouth's medium, supplemented with 15% fetal calf serum and containing 500 units of

penicillin and 500 μg streptomycin. In cultures fed from Day 2 onward, cell numbers plateau from Day 6 onward, at approximately 26 million cells per bottle. Cells grow as a monolayer, beginning exponential growth after 6 hr, with a doubling time of 12–14 hr. When cells from 2-day-old (exponentially growing) cultures were suspended in 0.04% Trypan Blue in Waymouth's medium, the percentage excluding the dye was routinely 98–100%. The plating efficiency of untreated control EMT-6 cells in vitro was 50–60%. The cells were harvested by centrifugation after rinsing with 2 ml of calcium- and magnesium-free Hanks' balanced salt solution, containing 0.05% trypsin EDTA (ethylenediaminetetraacetic acid). If the cells were to be counted for contained radioactivity, they were washed three times with nonradioactive Waymouth's medium, using centrifugation to remove surface-absorbed radioactivity. The mixture was centrifuged at 200g for 10 min at 20°C. The supernatant was removed and the cells resuspended in Waymouth's medium.

A number of Falcon tissue-culture flasks were prepared with 2.5×10^5 cells per flask. Cells were incubated at 37° in 5 ml of Waymouth's medium. Forty-eight hours later, 0.5 μCi of Ga-67-citrate and 0.5 ml of sterile Waymouth's medium were added to each tube. In addition, varying amounts of human transferrin* were also added to each tube. All samples were run in quadruplicate. The culture flasks were then incubated at 37°C. Cellular fractions were harvested at 24 hr after addition of the Ga-67-citrate. Final cell counts were determined and the amount of radioactivity in each flask was assayed, for the cellular fraction and for the incubation medium plus cell washings. Since the total volume of the cellular phase was 30.96 μl (or 0.563% of the total volume of cells plus medium), the concentration gradient of cells to medium was calculated by dividing the observed percentage uptake per 10 million EMT-6 tumor cells by 0.563%.

Human transferrin was labeled with I-125 using a lactoperoxidase (LPO) technique (15), using human transferrin (hTF) and "high-specific-activity, low-pH, Na^{125}I." Reaction yield was checked with 20% trichloroacetic acid, and precipitation was greater than 90%. Specific activity was about 15 μCi of I-125 per microgram human transferrin.

To measure cellular binding of I-125 transferrin as a function of hTF in the medium, I-125 transferrin (0.5 μCi per flask) was added to the cell-culture system, either in place of, or along with, the 0.5 μCi of Ga-67 citrate. After harvesting the cells, the relative cellular uptake of I-125 was assayed as described above for Ga-67 uptake.

In order to calculate binding parameters of the interaction between EMT-6 cells and transferrin (K,

equilibrium association constant; and n, number of receptor units per cell), we followed a kinetic approach used by Arend and Mannik (16) to determine binding parameters for the interaction between IgG and a macrophage receptor. The molecules of transferrin bound to one cell (r) and the molecules of transferrin free in the surrounding medium (c) were calculated for each experimental point using Avogadro's number and the molecular weight of 77,000 (17) for transferrin. A Scatchard plot of the form r/c = nK − rK was drawn, where K is the intrinsic association constant at equilibrium and r is the number of receptor units that bind transferrin (18). The value of n was determined as the x intercept, since this is just the number of receptor sites. The slope of the line was equal to −K. To construct each curve, the experimental data were fitted with a straight line by the least-squares method and a correlation coefficient was determined.

We also studied the binding between carrier-free Ga-67 and human serum transferrin. A detailed description of the methods of measurement is published elsewhere (19). From five determinations of K by this method, the range was $(2 \text{ to } 10) \times 10^5$ l/mole with an average of 4.89×10^5 l/mole.

Knowledge of K allows us to calculate the concentration of human transferrin at a given b (bound fraction of Ga-67) from the equation:

$$b/(1 - b) = \frac{B}{F} = K([TF] - b[Ga_t]) \qquad (1)$$

$$[TF] = \frac{b/(1 - b) + bK\,[Ga_t]}{K}, \qquad (2)$$

where [TF] = molar concentration of human transferrin in the medium. For b ranging between 0.05 and 0.95, the TF was calculated. For ease of comparison with the experimental results of other tissue-culture studies, TF was expressed as milligram of human transferrin per milliliter of medium. The results are shown in Fig. 4.

RESULTS

Figure 1 shows the effect of transferrin on Ga-67 uptake by EMT-6 tumor cells in vitro. The percentage of added Ga-67 that was concentrated in the cellular phase is shown on the left-hand vertical scale, and the ratio of cell concentration to medium concentration is shown on the right-hand scale. In the absence of added transferrin, there was negligible concentration of Ga-67 in the cellular phase compared with that in the medium. This was reflected by a cell-to-medium concentration ratio of approximately 1, corresponding to a Ga-67 uptake percentage of $0.5\%/10^7$ cells. With the addition of transferrin, there was marked increase in concen-
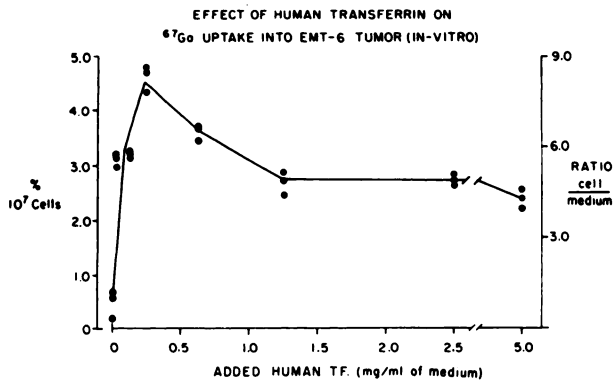
FIG. 1. Effect of added human transferrin on Ga-67 uptake into EMT-6 sarcoma growing in vitro. Ga-67 citrate (0.5 μCi) was added to exponentially growing EMT-6 tumor cells (≈ 10⁶) in 5.5 ml Waymouth's medium. Uptake with zero added transferrin was about 0.5%/10⁷ cells (cell-to-medium concentration ratio of about 1:1), but was significantly increased by the smallest increment in transferrin concentration (0.025 mg/ml).

tration gradient, so that cell-to-medium ratios of 5–6 were observed even at the lowest concentrations of added transferrin. This potentiation peaked at 0.25 mg transferrin per milliliter of medium, where the ratio of cellular concentration to medium concentration was about 8. Thereafter increasing amounts of transferrin caused a fall-off in Ga-67 uptake. At the highest concentrations studied (5 mg transferrin per milliliter of medium), there was still appreciable Ga-67 concentration, with a cell-to-medium ratio of 4.0.

Experiments were designed to evaluate the effect of hTF on the cellular uptake of I-125-labeled transferrin (I-125 hTF). The results are shown in Fig. 2.
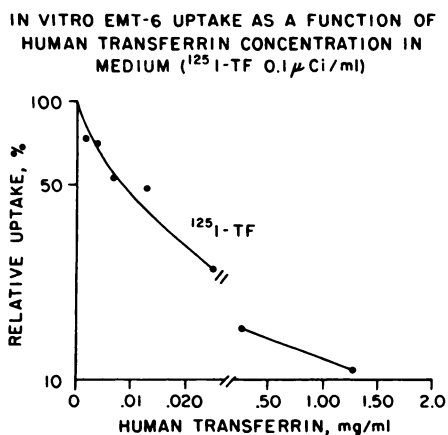

FIG. 2. Effect of added human transferrin (hTF) on I-125 hTF uptake into EMT-6 sarcoma. Incubation and experimental conditions were as in Fig. 1 except that 0.5 μCi I-125 hTF were added to each culture flask, and lower concentration of hTF was used. Uptake at zero added transferrin was taken to be 100%. Lowest transferrin increment gave a concentration of 1.6 μg/ml. Uptake of I-125 hTF is nearly saturated at 0.025 mg/ml, although there is some gradual displacement at higher concentrations.
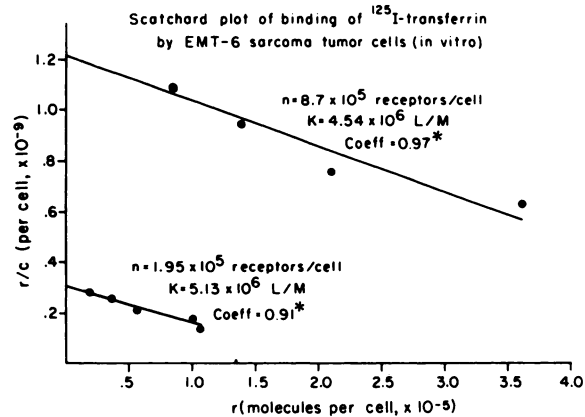
*Correlation coefficient over entire range of data points

FIG. 3. Scatchard plot of binding of I-125-labeled human transferrin to EMT-6 tumor cells growing in tissue culture. r = number of transferrin molecules bound per cell; c = number of unbound transferrin molecules. Plot of r/c vs r is nearly linear. Two sets of experimental data are shown. Association constant, K, and number of receptor units per cell, n, can be calculated from: $r/c = K(n - r)$.

The data for I-125 hTF uptake were normalized so that the uptake at the lowest concentration point (zero added TF) was taken to be 100%. As the transferrin concentration in the medium increased, there was a reduction in the cellular uptake of I-125 hTF to 1/10 of the original value. When the binding data from this and other experiments were subjected to Scatchard analysis, binding affinity (K), and the number of receptor sites (n), for the binding of I-125 hTF by EMT-6 cells could be calculated. In this analysis, r, the number of transferrin molecules bound per cell, was plotted against the ratio r/c, where c is the number of unbound transferrin molecules. Figure 3 shows that the relationship between r and r/c is well represented by a straight line. In these experiments, K had a relatively small variation. The number of transferrin receptor sites, n, showed relatively more variation. Data from two experiments are shown. For five experiments, the average K was 5×10^6 l/mole, and the number of receptor sites averaged 5×10^5 per cell.

Figure 4 shows the relationship between b, the fraction of Ga-67 bound to transferrin, and the concentration of human transferrin in Waymouth's medium; b was 0.10 at 0.01 mg/ml, and 0.50 at 0.08 mg/ml. A b of 0.9 was not reached until the transferrin was increased to 0.8 mg/ml, and 0.96 was not reached until 2.3 mg/ml (not shown on graph). The b was principally determined by the [TF], since [$^{67}Ga_t$] was very small. The effect on the calculation of varying [Ga_t] from 10^{-10} l/mole (dashed line) to 10^{-7} l/mole (solid line) was undetectable. Since the true concentration of [Ga_t] is in the range of 10^{-11} l/mole, its effect on the calculation of b was negligible.

Fraction of ^{67}Ga as ^{67}Ga-TF at

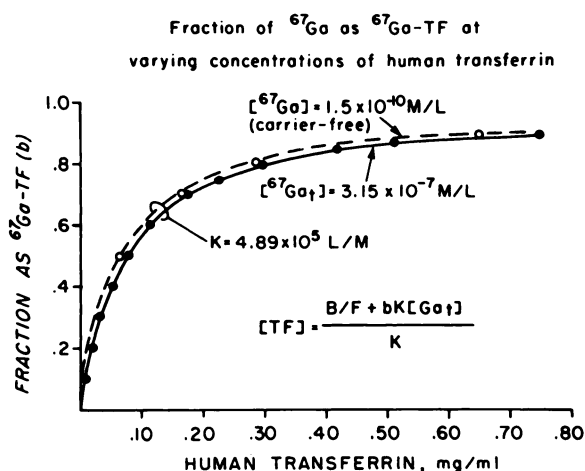varying concentrations of human transferrin

FIG. 4. Proportion of Ga-67 that is transferrin-bound at varying concentrations of transferrin in Waymouth's medium, when association constant, K, is 4.89 × 10⁵ l/mole.

We evaluated the hypothesis that Ga-67 is taken up into EMT-6 sarcoma tumor cells as Ga-67 labeled to transferrin. We reasoned that if this were occurring, the observed Ga-67 uptake should be proportional to the fraction of Ga-67 present as Ga-67 TF (Fig. 4) and the cellular uptake of transferrin as determined from I-125 transferrin studies (Fig. 2). Stated mathematically, our hypothesis becomes:

$$G(T) \; \alpha \; [X(T) \cdot Y(T)],$$

where G(T) = Ga-67 in cells; X(T) = Ga-67 as Ga-67 TF; and Y(T) = TF in cells, all three as functions of transferrin concentration in the medium. The product [X(T)·Y(T)] we call the "calculated cellular uptake."

We had available 69 individual Ga-67 uptake measurements from five different sets of experiments. These uptake measurements were performed over a range of four orders of magnitude in medium concentration of transferrin (10^{-4} mg/ml to 1 mg/ml). The uptake data at each concentration point were normalized to the average uptake percentage of Ga-67 in the culture flasks that contained zero added transferrin, for the five experiments. The normalized data points and a curve connecting the average values at each concentration step are shown in Fig. 5 as a solid line. The average percentage uptake in the absence of transferrin corresponded to a cell-to-medium concentration ratio of about 1. Therefore, the normalized number is actually the cell-to-medium Ga-67 concentration ratio. The curve reflects the change in this concentration ratio over four orders of magnitude of transferrin medium concentration. The average cell-to-medium Ga-67 concentration ratio peaks at about 12 at around 0.25 mg/ml human transferrin and declines as medium transferrin is increased further.

The "calculated cellular uptake" was based on the I-125 TF uptake data from a representative experiment, shown in Fig. 2; the fractional uptake of I-125 TF [Y(T)] at zero added transferrin was taken as 1.0. Where necessary, fractional I-125 TF binding values were obtained by extrapolation of the best-fit line between experiment values. The fraction of Ga-67 as Ga-67-TF[X(T)] was taken from Fig. 4. At various concentrations of transferrin the "calculated cellular uptake" was calculated as [X(T)·Y(T)]. This parameter was in turn normalized to the value at the lowest transferrin concentration, as follows. Since the zero concentration of added transferrin was calculated to have a X(T) = 0, this value could not be used for normalizing purposes. The problem was solved by considering the level of the first point for [X (T) · Y (T)] as having the same relative ratio of concentration as the corresponding Ga-67 uptake ratio, 1.1. Thus, the extreme lower end of the Ga-67 uptake curve and the "calculated cellular uptake" curve were matched.

Figure 5 shows that the shape of the Ga-67 uptake curve (solid) and "calculated cellular uptake" (dashed) were very similar, with a peak ratio at the same concentration of transferrin. As expected from this graph, the Ga-67 uptake measurements and the calculated cellular uptake parameter were highly correlated. By the least-squares method of linear correlation, an equation of G(T) = 0.66 [X(T)·Y(T)]-0.77, with r = 0.86 and p < 0.0001, was obtained for the relationship between the normalized ratios.
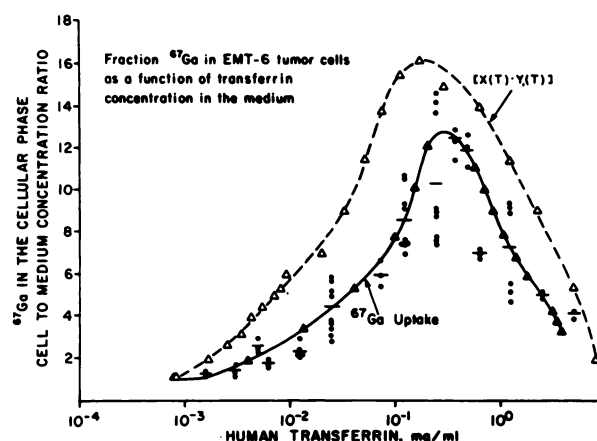

FIG. 5. Comparison between "calculated cellular uptake" [X(T)·Y(T)] (dashed line) and Ga-67 uptake (solid line). Data have been normalized so that lowest points on each curve match. Dots represent individual experimental values for Ga-67 cellular uptake. As in Fig. 1, there is peak stimulation of Ga-67 uptake at about 0.25 mg/ml added transferrin, with decline thereafter. Similar curve is found for "calculated cellular uptake."

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DISCUSSION

We believe that our uptake data support the viewpoint that transferrin and gallium are actually incorporated into the tumor cell, probably intracellularly. In the presence of transferrin, there is actual concentration of Ga-67 by the cells (i.e., cell-to-medium ratios ≫ 1), and a dilute solution of trypsin (used to harvest the cells) does not wash off the radioactivity. Membrane-bound radioactivity, even protein-bound radioactivity, should be removed by this process. In any event, whether inside or on the surface, there is actual cellular "uptake" of significant quantities of Ga-67, and furthermore this uptake is a function of transferrin concentration in the medium.

Recent evidence suggests an important role for a specific transferrin receptor in the uptake of iron by reticulocytes and other tissues (20,21). The transferrin-iron complex binds to a receptor on the cell membrane, and then the entire transferrin complex is actually taken into the cell by a process termed "adsorptive endocytosis." Once inside the cell, the iron is dissociated from the transferrin and bound to other molecules, e.g., hemoglobin, ferritin, etc. In hematopoietic tissues, the iron-free transferrin is then released back into the extracellular fluid.

The data from our experiments are consistent with an "adsorptive endocytosis" mechanism for the uptake of Ga-67 by EMT-6 cells. The importance of transferrin, the specific receptor, and the cellular concentration of I-125 TF by the cells were evidence in support of this mechanism (a cell-to-medium concentration ratio of about 3:1 for I-125 TF was achieved). Aulbert (22) has suggested that Ga-67 uptake by liver cells is due to endocytosis of Ga-67 transferrin complexes. He based his hypothesis on the discovery of Ga-67 labeled to transferrin both within the cytoplasm and associated with lysozomes. On the basis of our data, we can now develop a more complete hypothesis of the mechanism of gallium uptake, which incorporates some of Aulbert's ideas and is applicable to tumor tissue. Gallium-67 transferrin is taken up into EMT-6 tumor cells by adsorptive endocytosis. Unlike endocytosis in the usual sense, adsorptive endocytosis is a specific process, and in the case of Ga-67 transferrin, the process of incorporation into the cell is set in motion by the binding of the transferrin complex to a specific cellular receptor.

The work of Sephton and Harris (12,13) has been an important stimulus to our work. Our experiments have confirmed their findings that transferrin enhances Ga-67 uptake. We have extended these observations to a study of the transferrin receptor and its role in Ga-67 uptake. In our model system, the degree of Ga-TF complex formation is very important to the cell concentration of Ga-67 relative to medium, and such a mechanism may well account for the differences in uptake that they observed between Ga-67 and Fe-59 uptake as a function of transferrin concentration (13). In their system, the Fe-59 uptake increased to a peak at about 20 μg/ml added human transferrin, and declined thereafter. The Ga-67 uptake, on the other hand, increased to a plateau, with the highest value at 200 μg/ml. At 20 μg/ml, only about 10-15% of the Ga-67 would be expected to be transferrin bound (see Fig. 4). The Fe-59, which is much more avidly bound to transferrin, is probably almost totally in the form of Fe-59 TF at 20 μg/ml transferrin medium concentration. The decline in Fe-59 intracellular activity as transferrin increased further is probably the result of progressive saturation of the cell-associated transferrin receptor. The Ga-67 uptake continues to increase above this transferrin concentration because the rate of Ga-67 complex formation is more rapid than the displacement of transferrin from the receptor. A point is reached, however (and in our system it was at 200 μg/ml, the highest concentration point of Sephton and Harris), at which the Ga-67 TF complex formation curve (Fig. 4) begins to plateau, and at concentrations above this point, progressive saturation of transferrin receptor binding sites results in a decline in Ga-67 uptake (Figs. 1 and 5). If Sephton and Harris had extended their observations above 200 μg/ml, they probably would have noted a decline in Ga-67 uptake.

CONCLUSION

In conclusion, our in vitro studies of the uptake of Ga-67 into EMT-6 cells provided evidence that it was the Ga-67-transferrin complex that was concentrated by the cell. In the absence of added transferrin, Ga-67 was found in the cellular fraction, but it was not concentrated relative to the medium—the cell-to-medium concentration ratio was about one. When transferrin was added to the medium, true cellular "uptake" occurred, involving a cell-to-medium concentration gradient. Furthermore, the cellular uptake of Ga-67 transferrin was apparently mediated by a specific cellular receptor site for transferrin.

FOOTNOTE

* Sigma, Grade B, greater than 90% iron-free.

REFERENCES

1. EDWARDS CL, HAYES RL: Tumor scanning with ⁶⁷Ga citrate. *J Nucl Med* 10: 103-105, 1969
2. EDWARDS CL, HAYES RL: Scanning malignant neoplasms with ⁶⁷Gallium. *JAMA* 212: 1182-1190, 1970
3. VAIDYA SG, CHAUDRI MA, MORRISON R, et al: Localization of Gallium-67 in malignant neoplasms. *Lancet* 2: 911-914, 1970
4. WINCHELL HS, SANCHEZ PD, WATANABE CK, et al: Visualization of tumors in humans using ⁶⁷Ga citrate and the Anger whole-body scanner, scintillation camera and tomographic scanner. *J Nucl Med* 11: 459, 1970
5. PINSKY SM, HOFFER PB, TURNER DA, et al: Place of ⁶⁷Ga in staging of Hodgkin's disease. *J Nucl Med* 12: 385, 1971 (abst)
6. LANGHAMMER H, GLAUBITT G, GREVE SF, et al: ⁶⁷Ga for tumor scanning. *J Nucl Med* 13: 25-30, 1972
7. JOHNSTON GS, BENUA RS, TEATES CD, et al: ⁶⁷Ga-citrate imaging in untreated Hodgkin's disease: preliminary report of cooperative group. *J Nucl Med* 15: 399-403, 1974
8. GREENLAW RH, WEINSTEIN MB, BRILL AB, et al: ⁶⁷Ga imaging in untreated malignant lymphoma: Preliminary report of cooperative group. *J Nucl Med* 15: 404-407, 1974
9. DELAND FH, SAUERBRUNN BJL, BOYD C, et al: ⁶⁷Ga-citrate imaging in untreated primary lung cancer: Preliminary report of cooperative group. *J Nucl Med* 15: 408-411, 1974
10. JOHNSTON GS, GO MG, BENUA RS, et al: ⁶⁷Ga-citrate imaging in Hodgkin's disease: Final report of cooperative group. *J Nucl Med* 18: 692-698, 1977
11. ROCKWELL SC, KALLMAN RF, FAJARDO LF: Characteristics of a serially transplanted mouse mammary tumor and its tissue culture adapted derivative. *J Natl Cancer Inst* 49: 735-749, 1972
12. SEPHTON RG, HARRIS AW: Gallium-67 citrate uptake by cultured tumor cells stimulated by serum transferrin. *J Natl Cancer Inst* 54: 1263-1266, 1974
13. HARRIS AW, SEPHTON RG: Transferrin promotion of ⁶⁷Ga and ⁵⁹Fe uptake by cultured mouse myeloma cells. *Cancer Res* 37: 3634-3638, 1977
14. GAMS RA, WEBB J, GLICKSON JD: Serum inhibition of in-vitro ⁶⁷Ga binding by L1210 leukemic cells. *Cancer Res* 35: 1422-1426, 1975
15. THORELL JI, JOHANSSON BG: Enzymatic iodination of polypeptides with ¹²⁵I to high specific activity. *Biochim Biophys Acta* 251: 363-369, 1971
16. AREND WP, MANNIK M: The macrophage receptor for IgG: Number and affinity of binding sites. *J Immunol* 110: 1455-1463, 1973
17. SUTTON MR, MACGILLIVARY RTA, BREW K: Structural studies of human transferrin. In *Proteins of Iron Storage in Biochemistry and Medicine*, RR Crighton, ed. Amsterdam, North Holland/American Elsevier, 1975, pp 17-25
18. SCATCHARD G: The attractions of proteins for small molecules and ions. *Ann NY Acad Sci* 51: 660-672, 1949
19. LARSON SM, ALLEN DR, RASEY JS, et al: Kinetics of binding of carrier-free ⁶⁷Ga to human transferrin. *J Nucl Med* 19: 1245-1249, 1978
20. HEMMAPLARDH D, MORGAN EH: The role of endocytosis in transferrin uptake by reticulocytes and bone marrow cells. *Br J Haematol* 36: 85-96, 1977
21. BOCKXMEER FM, MORGAN EH: Identification of transferrin receptors in reticulocytes. *Biochim Biophys Acta* 468: 437-450, 1977
22. AULBERT E, GEBHARDT A, SCHULZ E, et al: Mechanism of ⁶⁷Ga accumulation in normal rat liver lysosomes. *Nuklearmedizin* 15: 185-194, 1976

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