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Serum Inhibition of *in Vitro* ^{67}Ga Binding by L1210 Leukemic Cells[1]

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SUMMARY

The influence of human serum on *in vitro* ^{67}Ga uptake by L1210 leukemic lymphoblasts has been investigated. Both high- and low-molecular-weight serum components inhibit cellular uptake of the isotope. Inhibition by the high-molecular-weight serum fraction correlates closely with the extent of binding of the radionuclide. Although transferrin participates in high-molecular-weight inhibition, it accounts for 10% or less of the inhibitory and binding capacity. Similarly, various low-molecular-weight serum components, including citrate, phosphate, glutamate, and others, contribute to inhibition. This inhibition of ^{67}Ga uptake by serum results from the presence of several, perhaps many, inhibitory components.

INTRODUCTION

The use of ^{67}Ga as a tumor-scanning agent (4) has stimulated investigation of the serum transport of this nuclide. Hartman and Hayes (13, 14) demonstrated *in vitro* binding of the radionuclide to proteins in human, rat, and rabbit sera. Using cross-electroimmunodiffusion of the serum obtained from patients after i.v. injection of ^{67}Ga, Gunasekera *et al.* (11) concluded that transferrin, haptoglobin, and albumin are the predominant serum protein binders of the isotope. However, immunoelectrophoretic studies by Clausen *et al.* (3) indicated that, apart from the weak binding of ^{67}Ga to β-lipoprotein, the metal was completely associated with transferrin. Farrer and Saha (5) demonstrated the delivery of ^{67}Ga to reticulocytes by transferrin and the possible incorporation of the metal into hemoglobin. NMR[3] and UV spectral studies by Woodworth *et al.* (19) demonstrated that 2 gallium(III) atoms are bound per molecule of conalbumin (a protein closely related to transferrin) and that the metal is associated with 4 tyrosyl phenolic ligands. Harris *et al.* (12) confirmed the presence of 2 gallium(III)-binding sites on transferrin. Gallium(III) therefore appears to bind to the iron(III)-binding sites on transferrin and conalbumin. ^{13}C NMR studies (12) indicate that carbonate is bound to transferrin within about 9 Å of the metal-binding site.

The recent observation by Fletcher *et al.* (7) that altered plasma binding of ^{67}Ga following chemotherapy or radiation significantly affects tumor deposition of the isotope suggests that serum components may influence cellular uptake of the isotope. *In vitro* studies in this laboratory (9, 10) demonstrated that serum and various buffers inhibit binding of ^{67}Ga by mouse L1210 leukemic lymphoblasts. These observations suggest that binding of the nuclide by serum components may render it unavailable for association with tumor cells. This hypothesis is consistent with enhanced *in vivo* tumor localization of the radionuclide following preinjection of scandium(III) (15) or iron(III) (5) metals which may preempt some gallium(III)-binding sites in plasma. In this study we have examined in greater detail the phenomenon of serum inhibition of *in vitro* ^{67}Ga uptake and its relationship to binding of this metal by serum components. This information may lead to a better understanding of the molecular mechanism of plasma transport of ^{67}Ga and its incorportion into neoplastic cells.

MATERIALS AND METHODS

^{67}Ga Samples. Stock solutions of ^{67}Ga citrate (carrier free) (New England Nuclear, Boston, Mass) containing 2 mCi/ml at noon of the day of delivery and 2 mg sodium citrate per ml were used within 2 weeks of the delivery date ($t_{1/2}$, 78.1 hr). Dilutions of this isotope were made in 0.9% NaCl solution and counted before use. Radioactivity was determined in an automatic well scintillation counter (Model 1185; Nuclear-Chicago Corp., Des Plaines, Ill.) previously calibrated with ^{67}Ga samples of known activity.

Serum Components. Human serum was obtained from normal volunteers. An aliquot of serum was dialyzed against a 500-fold excess of 0.9% NaCl solution for 24 hr at 0°. An Amicon UM10 membrane (10,000 M.W. cutoff) was used to prepare serum ultrafiltrate. Saturation of serum and apotransferrin (iron-free human transferrin) with iron(III) was accomplished with the nitrilotriacetic acid salt (Sigma Chemical Co., St. Louis, Mo.) according to the method of Bates *et al.* (2).

Purified preparations of apotransferrin, haptoglobin, and human serum albumin were purchased from Behring Diagnostics, Somerville, N. J. Low-molecular-weight components used in this investigation include trisodium citrate (American Chemical Society certified; Fisher Scientific Co., Pittsburgh, Pa.); lactic acid (American Chemical Society reagent; Matheson, Coleman & Bell, Norwood, Ohio); dextrose ("Baker analyzed" reagent; J. T. Baker

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[3] The abbreviation used is: NMR, nuclear magnetic resonance.

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Chemical Co., Phillipsburg, N. J.); urea (American Chemical Society certified; Fisher); adenosine, alanine, glutamic acid, and histidine (Schwarz/Mann, Orangeburg, N. Y.). Stock solutions were prepared in 0.9% NaCl solution by weight and the pH was adjusted to 6.8 ± 0.2 by the addition of hydrochloric acid or sodium hydroxide as required.

Serum Iron-binding Capacity. Serum unsaturated iron-binding capacity was determined by adding 100 μl of a solution of ferric ammonium citrate (50 mg/ml) containing ^{59}Fe (specific activity, 9×10^{-4} μCi/mg) to 1 ml of serum and determining the fraction bound by gel filtration on Sephadex G-25 (medium grade; Pharmacia Fine Chemicals, Inc., Piscataway, N. J.).

Cell-binding Studies. A slight modification of the *in vitro* ^{67}Ga binding procedure previously described (9) was used in these experiments. L1210 cells were harvested from the peritoneal cavity of female C57BL × DBA/2 F_1 mice 6 days after i.p. injection of 10^5 leukemic cells. The cells were washed 3 times with 0.9% NaCl solution and stored in an ice bath as a 4% packed cell volume (2.8×10^7 cells/ml) suspension. Duplicate samples (1 ml) of serum components were incubated for 1 hr at 37° in 12- x 75-mm Falcon 2052 counting tubes (Becton Dickinson, and Co., Oxnard, Calif.) with 2.60×10^{-5} μmole (1.05 μCi, 1.5×10^6 cpm) of ^{67}Ga (delivered in 100-μl aliquots); 250 μl of the cell suspension were then added and the incubation was continued for 2 additional hr. The reaction was then quenched by transferring the tubes to an ice bath and adding 2 ml of iced 0.9% NaCl solution to each tube. The cells were washed 3 times with iced 0.9% NaCl solution (4°) and the residual radioactivity was determined.

RESULTS AND DISCUSSION

While investigating *in vitro* binding of ^{67}Ga to L1210 leukemic lymphoblasts (9), we found that the presence of serum in the incubation mixture resulted in inhibition of cellular uptake of the isotope. Reports of gallium(III) binding by serum proteins (3, 11, 13, 14) suggested that this inhibition might result from the formation of complexes of the metal with high-molecular-weight serum components. We have also demonstrated by NMR spectroscopy that various buffers bind gallium(III) (8) and inhibit cellular uptake of ^{67}Ga (10). Since some of these, such as citrate, phosphate, and lactate, are normal serum components, the low-molecular-weight serum fraction might also have an inhibitory effect.

Inhibition by Serum Fractions. The influence of serum fractions on the *in vitro* uptake of ^{67}Ga by L1210 cells is shown in Table 1. As we have noted previously (9), serum markedly inhibits uptake of the isotope by L1210 cells in this *in vitro* test system. Dialysis of serum to remove low-molecular-weight components eliminates approximately one-half the inhibitory capacity. The inhibitory capacity of serum ultrafiltrate, which contains molecules with molecular weights of less than 10,000, is about equal to that of the high-molecular-weight serum fraction.

High-Molecular-Weight Serum Fraction. High-molecular-weight serum components were fractionated by gel filtration on Sephadex G-200 (Chart 1). The elution profile demonstrates 3 major protein peaks. Peak I, corresponding to the void volume of the column, contains substances the molecular weights of which are in excess of 200,000 (*e.g.*, IgM). Proteins with a molecular weight of approximately 160,000 (*e.g.*, IgG) elute in peak II. The molecular weight range of Peak III is approximately 75,000 to 100,000; transferrin, albumin, and haptoglobin are eluted largely in this peak (16). Each of these peaks (pooled and concentrated) inhibits *in vitro* uptake of ^{67}Ga by L1210 cells. Peak II has the greatest inhibitory capacity (84%), although Peak I (39%) and Peak III (59%) also inhibit significantly. It is apparent from this experiment that inhibition of cellular ^{67}Ga uptake appears to be caused by at least 3 high-molecular-weight serum components.

Table 1
Inhibition by serum fractions of ^{67}Ga uptake by L1210 cells

Incubation medium	^{67}Ga uptake (μCi/10^6 cells)	% inhibition[a]
0.9% NaCl solution	1.41	0
Whole serum (1:10 dilution)	0.16	89
Dialyzed serum (1:10 dilution)	0.74	47.6
Ultrafiltrate of serum (1:10 dilution)	0.75	46.7

[a] $(C_0 - C)/C_0$ (C_0, ^{67}Ga uptake in 0.9% NaCl solution; C, ^{67}Ga uptake in the test solution).

Table 2 compares the inhibitory capacities of high-molecular-weight serum components with that of some purified serum proteins. Transferrin, albumin, and haptoglobin were investigated because of previous reports implicating these proteins as serum binders of ^{67}Ga (3, 11, 13). Concentrations of these purified proteins approximated those found in normal serum (18). Albumin and haptoglobin do not inhibit cellular uptake of the isotope. Although transferrin does inhibit binding of ^{67}Ga by L1210 cells, it does so to a lesser extent than does dialyzed serum. Furthermore, transferrin inhibition is reduced by 82% after saturation of the protein with iron, whereas the inhibitory capacity of dialyzed serum is reduced only 16% by iron saturation. This suggests that transferrin accounts for only a small part of the inhibitory capacity of the high-molecular-weight serum fraction.

Binding of ^{67}Ga to high-molecular-weight serum components was determined by gel filtration on Sephadex G-25 (Chart 2). Two peaks of radioactivity were obtained. The 1st coincided with the protein peak in the void volume and contains substances with molecular weights in excess of 5000. The 2nd peak coincided with the elution volume of free ^{67}Ga. This method is sensitive only to relatively strong binding since weakly associated ^{67}Ga will dissociate during passage through the column.

The amount of ^{67}Ga tightly bound to high-molecular-weight serum components is summarized in Table 3. Albumin and haptoglobin bind very little ^{67}Ga. This is consistent with the inability of these proteins to inhibit cellular uptake of the isotope. The extent of binding of ^{67}Ga by dialyzed serum and apotransferrin is comparable to the

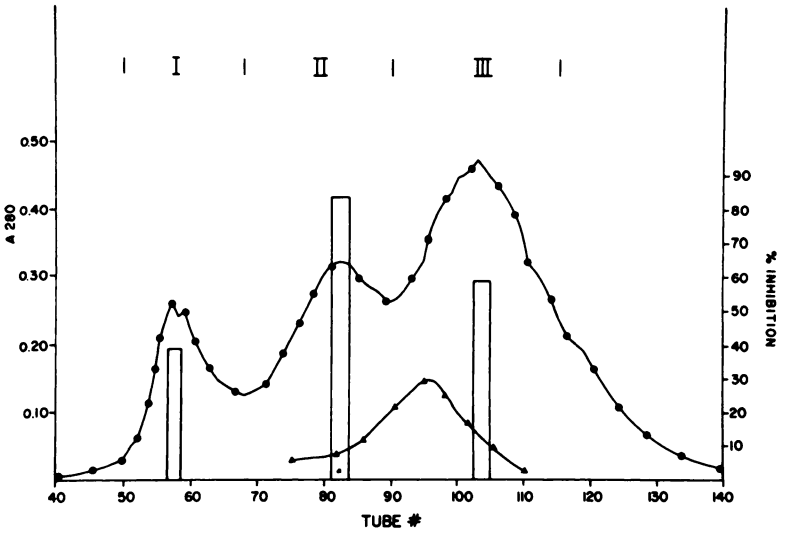

Chart 1. Elution profile of dialyzed whole serum (●) and apotransferrin (20 mg/ml) (▲) on Sephadex G-200 (column size, 2.6 x 70 cm; sample size, 4 ml; eluant, 0.9% NaCl solution; fraction size, 2.0 ml; flow rate, 12 ml/hr). Each major protein peak (*I*, *II*, *III*) of whole serum was concentrated to 4 ml by ultrafiltration and tested for ability to inhibit cellular uptake of ^{67}Ga (*bars*).

Table 2

Inhibition by high-molecular-weight serum components of ^{67}GA uptake by L1210 cells

Incubation medium	^{67}Ga uptake (μCi/10^6 cells)	% inhibition[a]
0.9% NaCl solution	1.41	0
Dialyzed serum (undiluted)	0.18	87.1
Dialyzed iron-saturated serum[b]	0.37	73.9
Apotransferrin[c] (2 mg/ml)	0.62	55.7
Transferrin[d] (2 mg/ml)	1.26	10.0
Albumin (30 mg/ml)	1.41	0
Haptoglobin (1.5 mg/ml)	1.41	0

[a] See Table 1.
[b] Serum saturated with iron nitrilotriacetic acid (iron, 10 μg/ml serum) and excess iron removed by dialysis.
[c] Iron free.
[d] Iron saturated.

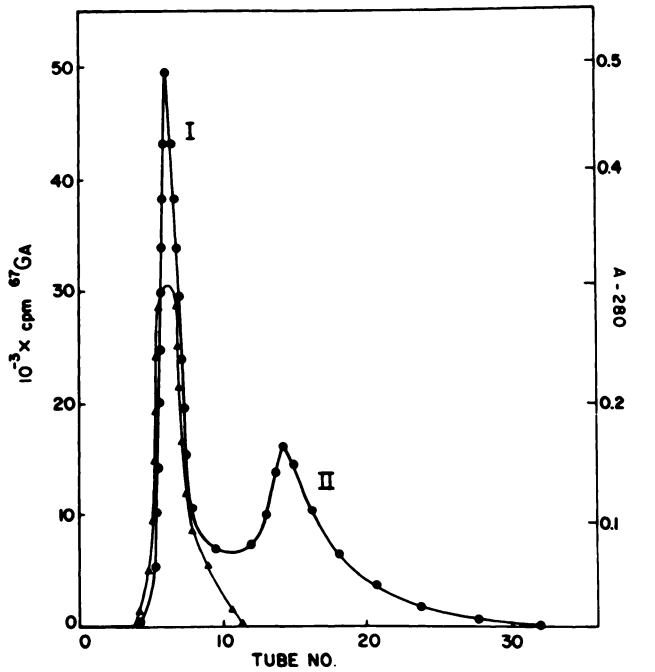

Chart 2. Elution profile of dialyzed serum (1:10 dilution) preincubated with ^{67}Ga on Sephadex G-25 (column size, 0.9 x 25 cm; sample size, 1.0 ml; eluant, 0.9% NaCl solution; fraction size, 1.0 ml; flow rate, 6 ml/hr). ●, radioactivity; ▲, A_{280}.

ability of each of these to inhibit cellular uptake of the isotope (compare Tables 2 and 3). Binding of ^{67}Ga by apotransferrin is almost completely eliminated by saturation of the protein with iron(III). This effect is consistent with both metals competing for the same site on the protein, as has been suggested by Hartman and Hayes (13) and Woodworth *et al.* (19). The similar ionic radii [gallium(III), 0.62 Å; iron(III), 0.64 Å] and charges of these ions may account for this behavior. Clausen *et al.* (3) have also investigated binding of ^{67}Ga by transferrin. They concluded that there were 14 gallium(III)-binding sites per mole of protein with an equilibrium constant of 1.772 liters/mole. Such a small binding constant reflects predominantly adventitious binding of the metal. It has previously been demonstrated that apotransferrin will bind iron(III) adventitiously unless the iron(III) is administered with a strong ligand such as nitrilotriacetic acid (2). In the latter instance transferrin is found to have only 2 binding sites for iron(III) with a stability constant of about 10^{37}. It seems likely, therefore, that gallium(III) occupies both the iron-binding sites on apotransferrin as well as other weaker binding sites on the molecule. In the presence of several classes of binding sites, the simple Scatchard analysis used by Clausen *et al.* (3) is no longer valid. A generalized Scatchard analysis (6) could have been used to obviate this difficulty. Adventitious binding of ^{67}Ga may account for the residual 10% inhibitory effect of apotransferrin after saturation with iron(III) (Table 2).

Chart 3 demonstrates the close correlation between binding of ^{67}Ga and inhibition of cellular uptake of the isotope by serial dilutions of dialyzed human serum and purified human apotransferrin. Apotransferrin concentrations were computed from the unsaturated iron-binding capacity of each serum dilution. Although the shapes of the 2 curves are nearly identical, the apotransferrin curve is

shifted to higher concentrations by approximately 1 log order. This indicates that purified apotransferrin is only about one-tenth as effective as dialyzed whole serum in binding ^{67}Ga or inhibiting the uptake of the radioisotope by L1210 cells.

Low-Molecular-Weight Serum Components. Sarkar (17) has identified citrate as the major iron(III)-binding low-molecular-weight serum component. This anion may also be expected to be a major serum binder of gallium(III). Table 4 shows the influence of representative low-molecular-weight serum components on *in vitro* ^{67}Ga uptake by L1210 cells. Although citrate is the strongest inhibitor of tumor localization of the metal, its inhibitory capacity does not approach that of serum ultrafiltrate. Thus, various low-molecular-weight serum components contribute to the inhibitory effect of serum. As in the case of inhibition by high-molecular-weight serum components, low-molecular-weight components appear to inhibit by forming gallium(III) complexes that compete with tumor cells for available ^{67}Ga. Formation of both low-molecular-weight and polymeric gallium(III) citrate complexes has been demonstrated by NMR and dialysis studies in our laboratory (8). Using NMR spectroscopy, we have also detected complexes of gallium(III) with lactate and phosphate (F. Chang, T. P. Pitner, R. A. Gams, J. D. Glickson, unpublished data). The ability of adenosine and dextrose to enhance cellular uptake of ^{67}Ga is intriguing. These agents may serve as carriers of the metal or may influence its uptake through their effect on cellular metabolism of the isotope. These possibilities are presently under study.

Both high- and low-molecular-weight serum components can inhibit the cellular uptake of ^{67}Ga. While the most reasonable explanation for this phenomenon appears to be the formation of tumor-impermeable gallium complexes, we cannot exclude the possibility that these components may interact with cell surface components or modify cellular metabolism. Serum inhibition of ^{67}Ga uptake cannot be explained by the presence of any 1 inhibitory component. Rather, inhibition appears to result from the presence of several, perhaps many, components, some of which may be present in trace quantity. Consequently, it may be very difficult to identify these inhibitors. Although apotransferrin participates in this process, our data suggest that it can account for only 10% or less of the inhibitory capacity of the high-molecular-weight serum fraction.

Table 3

Binding of ^{67}Ga by serum components

Samples (1 ml) were incubated for 1 hr with 1.05 μCi ^{67}Ga citrate delivered in 100 μl 0.9% NaCl solution and subjected to gel filtration on Sephadex G-25.

Serum component	% bound[a]
Dialyzed serum	85
Albumin (30 mg/ml)	5
Haptoglobin (1.5 mg/ml)	2
Apotransferrin[b] (2 mg/ml)	49
Transferrin[c] (2 mg/ml)	2

[a] 100 × (total cpm in protein peak/total cpm eluted from column).
[b] Iron free.
[c] Iron saturated.

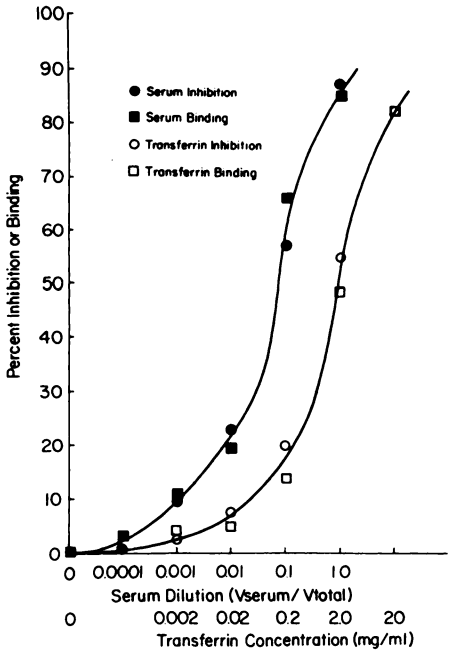

Chart 3. Binding of ^{67}Ga and inhibition of its cellular uptake by serial dilutions of dialyzed human serum and apotransferrin.

Table 4

Inhibition by low-molecular-weight serum components of ^{67}Ga uptake by L1210 cells

Incubation medium[a]	^{67}Ga uptake (μCi/10^8 cells)	% inhibition[b]
0.9% NaCl solution	1.41	0
Serum ultrafiltrate (undiluted)	0.16	88.3
Citrate (3.2 mg/dl)	0.94	33.0
Phosphate (9.6 mg/dl)	0.99	30.0
Glutamate (3.4 mg/dl)	1.02	28.0
Lactate (16.9 mg/dl)	1.23	13.0
Urea (27 mg/dl)	1.25	11.0
Alanine (4 mg/dl)	1.28	9.0
Histidine (1.5 mg/dl)	1.41	0
Dextrose (100 mg/dl)	1.76	−25
Adenosine (1 mg/dl)	1.83	−30

[a] The approximate concentrations of these components in normal human serum have been used (1).
[b] See Table 1.

It has been proposed that enhanced tumor localization of ^{67}Ga by preinjection of scandium(III) (15) or iron(III) (5) results from displacement of gallium(III) from plasma binding sites. This would suggest that the inhibitory effect of serum that we have observed *in vitro* may play a role in *in vivo* tumor scanning. Plasma binding may serve to clear the isotope from tissues with low affinity for the nuclide, thus permitting scintigraphic observation of areas involved with tumor. The demonstration that some substances such as dextrose or adenosine may enhance cellular uptake of ^{67}Ga serves as a provocative stimulus to search for other substances that may have a similar effect. Clearly, optimum use of tumor-scanning agents such as ^{67}Ga requires an intimate understanding of the mechanisms of its localization

in malignant cells. These experiments have indicated that plasma probably plays an important role in this process, and studies to characterize it more thoroughly are presently under way in our laboratory.

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