Effects of Buffers and pH on in Vitro Binding of $^{67}$Ga by L1210 Leukemic Cells

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SUMMARY

The effect of sodium nitrate and a series of buffers on in vitro $^{67}$Ga binding to L1210 leukemic cells at pH 6.8 ± 0.2 and 37°C at concentrations of $10^{-7}$ to $10^{-2}$ M has been investigated. The relative ability of these agents to inhibit cellular incorporation of $^{67}$Ga is ethylenediaminetetraacetate $>$ nitrilotriacetic acid $>$ citrate $>$ bicarbonate $>$ phosphate, lactate, Tris $>$ morpholinopropane sulfonic acid (MOPS), N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid, nitrate $\approx$ 0. Inhibition probably results from formation of gallium (III) complexes which are either impermeable to the tumor membrane or which compete with intracellular receptor complexes. However, direct interaction of buffers with the cell membrane or with gallium (III) receptors, as well as effects of buffers on cellular metabolism, have not been excluded. A monotonic decrease in the cellular incorporation of $^{67}$Ga occurs between pH 6.2 and 7.8 in the presence of the inert buffer, $10^{-2} \text{M}$ morpholinopropane sulfonic acid.

INTRODUCTION

Preferential localization of $^{67}$Ga in a broad range of solid tumors and malignant lymphomas has been employed in the clinical detection and early staging of malignant disease by $^{67}$Ga scintigraphy (4, 11). An understanding of the detailed mechanism of $^{67}$Ga localization in tumor cells may lead to the rational development of optimum methods for the detection of malignant neoplasms and may also reveal some underlying differences between normal and transformed cells that determine their interaction with metal ions. To help attain these objectives, an in vitro technique for measuring binding of $^{67}$Ga by dispersed cells was recently developed in this laboratory (8). Preliminary experiments indicated that a number of buffers (bicarbonate, phosphate, Tris, barbital, maleic acid, and citrate) inhibit in vitro uptake of $^{67}$Ga by L1210 leukemic cells. This study examines buffer inhibition and pH effects in more detail. The need to study the effects of counterions on the incorporation of $^{67}$Ga by isolated tumor cells is underscored by recent experiments by Konikowski et al. (10), who report a significant difference in tumor localization and clearance of the citrate, lactate, and chloride salts of $^{67}$Ga as opposed to the DTPA $^3$ salt.

Materials and Methods

Buffer Inhibition. Stock solutions of $^{67}$Ga citrate and $^{67}$Ga nitrate (New England Nuclear, North Billerica, Mass.) containing 2 mCi/ml at noon of the day of delivery were used within 2 weeks of purchase ($t_{1/2} = 78.1$ hr). Commercial preparations of the citrate salt contained sodium citrate, 2 mg/ml. The nitrate sample was approximately 0.5 M in nitrate. Dilutions were made in 0.9% NaCl solution and counted in a Model 1185 automatic well scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.), that had been calibrated with $^{67}$Ga samples of known activity. Buffers and salts used in this investigation included sodium bicarbonate (J. T. Baker Chemical Co., Phillipsburg, N. J.); potassium dihydrogen phosphate (American Chemical Society certified; Fisher Scientific Co., Fair Lawn, N. J.); lactate (American Chemical Society reagent; Matheson, Coleman, and Bell, Norwood, Ohio); Tris (Fisher Scientific Co.; certified primary standard); morpholinopropane sulfonic acid (MOPS) (Sigma Chemical Co., St. Louis, Mo.); HEPES (Sigma); sodium nitrate (Baker; analyzed reagent); trisodium citrate (Fisher; American Chemical Society certified), EDTA disodium salt (Cambridge Chemical Products, Inc., Detroit, Mich.), and NTA (Sigma).

Except for modifications described below, the in vitro $^{67}$Ga-binding procedure described in detail elsewhere (8) was used. L1210 cells were harvested from the peritoneal cavity of female C57BL X DBA/2 F1 (hereafter called BD2F1) mice 6 days after i.p. inoculation of $10^5$ leukemic cells. The cells were washed 3 times with 0.9% NaCl solution and a 4% packed-cell volume (2.8 $\times$ $10^7$ cells/ml) suspension was prepared and stored in an ice bath. Samples of the buffer or salt (1 ml) adjusted to pH 6.8 ± 0.2 with HCl and/or NaOH were incubated in 12- x 75-mm Falcon 2052 counting tubes (Becton, Dickinson and Company, Oxnard, Calif.) for 1 hr at 37°C with 2.60 $\times$ $10^{-8}$ mmole (1.05 $\mu$Ci, 1.5 $\times$ $10^6$ cpm) of $^{67}$Ga (delivered in 100 $\mu$l of 0.9% NaCl solution). At specified time intervals, 250- $\mu$l aliquots of cell suspension were added to duplicate tubes and the incubation was continued. The reaction was 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 additional times with 0.9% NaCl solution (at 4°C), and the residual radioactivity was determined.

pH Studies. Cell-binding studies were performed as described above, with the use of an incubation medium consisting of 1 ml $10^{-2}$ M morpholinopropane sulfonic acid in 0.9% NaCl solution adjusted to pH 6.2, 6.6, 7.0, 7.4, and 7.8. The cell reaction was quenched after 2 hr of cell incubation.

Received May 13, 1974; accepted July 16, 1974.

This research was supported by Grant CA-13148 from the NIH.

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2The abbreviations used are: DTPA, diethylenetriamine-$N,N',N'',N'''$, $N''''$-pentacetic acid; HEPES, $N$-2-hydroxyethylpiperazine $N'$-2-ethane sulfonic acid; NTA, nitrilotriacetic acid.

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RESULTS

Chart 1 summarized the kinetics of in vitro binding to L1210 leukemic cells of 2 commercial preparations of carrier-free $^{67}$Ga, i.e., the citrate and nitrate salts. The $^{67}$Ga concentration was 1.93 X $10^{-11}$ M for both samples. Approximate concentrations of citrate and nitrate ions in the in vitro incubation mixture were 1.6 X $10^{-10}$ M and 1.2 X $10^{-10}$ M, respectively. Zinc, formed by radioactive decay of $^{67}$Ga, was estimated at a level of about 6.65 X $10^{-11}$ M. It has previously been demonstrated that zinc at this concentration does not influence the extent of in vitro $^{67}$Ga binding by L1210 leukemic cells (8). Incubation at 37° was limited to 2 hr, during which time the exclusion of the vital dye trypan blue suggests retention of membrane integrity of the L1210 cells (8). During this time interval, the cells responded in an identical manner to the citrate and nitrate salts of $^{67}$Ga (Chart 1).

Chart 2 shows inhibition curves for nitrate, HEPES, morpholinopropane sulfonic acid, Tris, lactate, phosphate, and bicarbonate. Nitrate, HEPES, and morpholinopropane sulfonic acid have no effect on in vitro $^{67}$Ga binding up to a concentration of 10$^{-2}$ M. Tris, lactate, and phosphate inhibit slightly at concentrations above 10$^{-3}$ M, whereas bicarbonate inhibits somewhat more. Citrate, NTA, and EDTA are much more potent inhibitors (Chart 3). Since the pH remained at 6.8 ± 0.2 after the 2-hr incubation, the inhibitory effects originate from the specific buffers used in these experiments, rather than from pH changes. The relative order of inhibitory capacity is EDTA > NTA > citrate > bicarbonate > phosphate, lactate, Tris > morpholinopropane sulfonic acid, HEPES, nitrate ≈ 0.

DISCUSSION

The aqueous chemistry of gallium (III) is complex and not completely characterized (2, 15). Salts such as the nitrate (18) and perchlorate (5, 6) dissociate completely in strongly acidic solution to yield Ga(H$_2$O)$_6$$^{3+}$ ions. However, the solubility product of Ga(OH)$_3$ (about 5 X 10$^{-19}$) (2) indicates that the maximum concentration of hydrated gallium (III) in neutral solution is about 5 X 10$^{-16}$ M. Consequently, gallium (III) is present in solution in the form of various complexes. The perchlorate salt forms metastable polymers with maximum degrees of polymerization near pH 3 (3, 14). Citrate polymers have also been detected in this laboratory by equilibrium

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*Concentrations of gallium (III) have been calculated from the μCi of $^{67}$Ga measured, assuming the radiochemical was carrier free; 1.05 μCi (2.60 X 10$^{-8}$ mmoles) of $^{67}$Ga were administered.
Given the context, the document appears to discuss the effects of buffers on the binding of $^{67}$Ga to L1210 leukemic cells. The text mentions that the inhibition of $^{67}$Ga binding by L1210 leukemic cells incubated with sodium citrate (○), NTA (○), or EDTA (△) at pH 6.8 ± 0.2 at 37°. Each point, average of duplicate measurements.

The shapes of the EDTA, NTA, and citrate inhibition curves (Chart 3) can readily be understood in terms of complex formation. These curves resemble titration curves for metal-complex formation. At high buffer concentrations, all the available metal may be sequestered in complexes unavailable for tumor localization. These complexes, however, would dissociate at low buffer concentrations by a mass law effect, thereby liberating $^{67}$Ga for tumor binding. Binding of $^{67}$Ga to L1210 cells may serve as a measure of(891,362),(963,469)

The inhibitory effect of the buffers, at least that of the chelating agents, does not result solely from competition for cell surface-bound $^{67}$Ga. In the absence of buffer, only about 12% of the cell-bound $^{67}$Ga appears to be adsorbed on the cellular membrane (8), whereas the inhibitory capacities of EDTA, NTA, and citrate approach 100%.

Inhibitory effects such as those described in these in vitro experiments may be significant in the design and interpretation of in vivo studies of $^{67}$Ga localization. Average normal concentrations of citrate in serum are about 7.5 × 10^{-5} M (19). This citrate concentration is just below the level at which significant inhibition of in vitro $^{67}$Ga uptake by L1210 cells is observed (Chart 3). The observation by Konikowski et al. (10) that under in vivo conditions citrate, lactate, and chloride salts of $^{67}$Ga localize to similar extents in tumors and are cleared at similar rates is consistent with the conclusion that citrate exerts a negligible inhibitory effect. However, these authors did observe a significant diminution in the extent of tumor localization of $^{67}$Ga when the DTPA salt was injected. Spectrophotometric titration studies indicate that the stability constant for gallium (III) DTPA is about $10^{-2.8}$ at 20° (12).
Such stable complexes may indeed inhibit tumor localization of $^{67}$Ga. The effect of pH was studied in order to explore the molecular mechanism of cellular $^{67}$Ga incorporation. In the limited pH range used in these studies, tumor uptake of the radioisotope increased dramatically with decreasing pH. Increasing uptake of the radioisotope in acidic media could be caused by: (a) stabilization of molecular states of $^{67}$Ga favorable for incorporation in the tumor, (b) titration of cellular receptors, or (c) decomposition of cellular membranes in acidic media. A definitive choice between these mechanisms requires detailed information about the structure of the tumor cell and the identification of the molecular species of gallium (III) localized within tumor cells. If it is assumed that the first effect, variations in the concentration of tumor permeable species of gallium (III), contributes, at least in part, to the observed variations of cellular uptake, the effect of pH could be used to help identify the species of this element which localize in L1210 cells. These would have to be species whose concentration increases significantly between pH 8 and 6. Additional data on the aqueous chemistry of gallium (III) is required to identify these species.

ACKNOWLEDGMENTS

The authors are indebted to Dr. Paul Saltman, Dr. S. Y. Tyree, and Dr. Phillip Aisen for helpful discussion of the data, to Dr. K. H. Kim and Mary Sabens for technical assistance, and to Dr. Frank M. Schabel and Mary W. Trader for the generous provision of L1210 leukemic BD2F, mice.

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\textit{Cancer Res} 1974;34:2957-2960.

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