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Transferrin Promotion of ^{67}Ga and ^{59}Fe Uptake by Cultured Mouse Myeloma Cells[1]

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SUMMARY

Radiotracer ^{67}Ga-citrate is used as a tumor-seeking agent in clinical imaging investigations although fundamental reasons for its high uptake in certain malignant lesions remain unexplained. The mechanism by which ^{67}Ga becomes concentrated in tumor cells has been investigated by comparing ^{67}Ga and ^{59}Fe uptake by cultured mouse myeloma cells with particular reference to uptake stimulation by transferrin. Concentrations of human transferrin down to 2 μg/ml greatly stimulated cellular uptake of both tracers, whereas bovine transferrin proved relatively inactive. The rates of stimulated uptake of both tracers were similar as was their high degree of retention by cells, but their quantitative dependencies on transferrin concentration showed characteristic differences. Pretreatment of human transferrin with saturating amounts of nonradioactive Fe^{3+} canceled its ability to promote ^{59}Fe uptake, but it had little effect on its promotion of ^{67}Ga uptake. Further increase in the amount of added Fe^{3+} did cause a progressive depression of ^{67}Ga uptake, but this effect probably relates to the iron distribution in the whole-cell culture system including the fetal calf serum component of cell growth medium. The results suggest that ^{67}Ga and ^{59}Fe reveal different aspects of the interaction of transferrin with cells.

INTRODUCTION

Since the discovery of its affinity for tumors (5), ^{67}Ga-citrate has been widely used in clinical nuclear medicine for the detection and location of malignant deposits (11, 12, 17). The general clinical experience has been that distinctive ^{67}Ga accumulation occurs particularly in lymphoid tumors and also in inflammatory lesions, but its mechanism is not understood. It is known that ^{67}Ga becomes firmly bound inside cells, and a predominantly lysosomal location has been reported (3, 16). Its binding to tissue proteins (4, 9) and serum proteins (4, 7, 8) has been studied; transferrin in particular was identified as one of the binding components (4, 7). Experimental studies *in vivo* have shown a relationship between uptake and growth rate for both bone marrow and transplanted myeloma tumors (2) and a marked reduction in tissue uptake after whole-body X-irradiation (15).

Several investigations of ^{67}Ga uptake by cells *in vitro* have been described, but these have mostly used short-term incubation of cells taken from animals (*e.g.*, Refs. 6, 10, and 13). In experiments with cells of mouse tumor lines grown in continuous culture, we have demonstrated that certain kinds of serum can promote cellular accumulation of ^{67}Ga (14). Although the culture medium routinely contained 10% fetal calf serum, additional rabbit, horse, or human serum at as low as 0.2% greatly increased ^{67}Ga uptake by cultured myeloma, T-lymphoma, or mastocytoma cells. The active component of both human and horse serum was shown to be transferrin (14).

Since transferrin is the principal iron carrier in serum and since some information as to its mechanism of iron transport into erythroid cells is already known (1), we have made some comparisons between ^{59}Fe and ^{67}Ga uptake by cultured tumor cells in dual tracer experiments. In this paper we report on some of the characteristics of transferrin-promoted ^{67}Ga and ^{59}Fe uptake by cultured cells of a mouse myeloma line.

MATERIALS AND METHODS

Cells. Mouse myeloma cell line HPC-108.1 was chosen because its growth in stationary suspension culture is rapid and reliable and its ability to concentrate ^{67}Ga is somewhat greater than that of several other cell lines tested (14). It originated in 1971 (A. W. Harris, M. Holmes, and N. L. Warner, unpublished data) as paraffin oil-induced tumor HPC-108 in the peritoneal cavity of a 12-month-old male BALB/c ↔ C57BL chimeric mouse (constructed by embryo aggregation). The tumor was transplanted once through a BALB/c mouse, was then adapted to growth in culture and, after several months of growth *in vitro*, was cloned by direct single-cell isolation to yield a line designated HPC-108.1. For the derivation of the cell line and the present experiments, the culture medium comprised Dulbecco's modified Eagle's medium [Powder H-16 (Grand Island Biological Co., Grand Island, N. Y., or Commonwealth Serum Laboratories, Parkville, Australia)] with 3.4 g $NaHCO_3$ per liter, 100 units penicillin G sodium per ml, 100 μg streptomycin sulfate per ml, and 10% heat-inactivated (56° for 1 hr) fetal calf serum (Commonwealth Serum Laboratories). Fetal calf serum from Batch 783 was used in all experiments described in this paper. In this medium in a 10% CO_2-air incubator at 37°, the cells grew exponentially, with a mean population-doubling time of about 12 hr and a mean cell volume of about

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1.5 pl, to a stationary phase at about 10^6 cells/ml. Cell concentrations and mean cell volumes were determined with a Model F_N Coulter counter (Coulter Electronics Ltd., Dunstable, England) and its accessory mean cell volume computer, which was calibrated with red mulberry pollen of a mean grain volume of 1.54 pl.

Radioactive Tracers. Carrier-free ^{67}Ga with a stated specific activity of >30 Ci/mg was obtained in citrated form (Radiochemical Centre, Amersham, England) and used in cell cultures at a concentration of about 0.1 μCi/ml. ^{59}Fe as ferric citrate (Radiochemical Centre), with a specific activity of 10 Ci/g, was used at about 0.01 μCi/ml. Within each experiment all cultures contained equal ^{67}Ga and equal ^{59}Fe concentrations although the concentrations varied moderately between experiments. Stock solutions of the tracers were diluted to appropriate concentrations in 0.85% NaCl solution and added to cultures within 1 hr of dilution.

Transferrins. Purified human and bovine transferrin (Calbiochem, Sydney, Australia) were dissolved in 0.85% NaCl solution and sterilized by filtration [GSWP filter (Millipore Corp., Bedford, Mass.)].

Iron Loading of Transferrins and Serum. Freshly prepared solutions of ferric citrate in water were sterilized by filtration, their iron content was measured by atomic absorption spectroscopy (Varian Techtron, Model AA5), and dilutions were added to solutions of human transferrin. These mixtures were incubated at 37° for 24 hr before their use in cell culture experiments. Iron loading of filtered serum was effected similarly.

Measurements of ^{67}Ga and ^{59}Fe Uptake by Cells. Suspensions of exponentially growing HPC-108.1 cells were diluted in warm culture medium to a concentration that would allow continued exponential cell growth through a subsequent incubation period with added tracers. To the diluted bulk cell suspension was added ^{67}Ga citrate, and then 5-ml aliquots were dispensed into 50-mm-diameter plain plastic Petri dishes (Kayline Medical Plastics, Adelaide, Australia) containing separate aliquots of ^{59}Fe citrate and transferrin or 0.85% NaCl solution (as appropriate) in volumes not exceeding 0.2 ml each. Duplicate cultures for each variable under test were set up. After incubation, each culture was mixed, a 0.2-ml sample was taken for cell concentration and mean cell volume determinations, and the remainder was centrifuged in conical plastic centrifuge tubes (Kayline) at 700 × *g* for 10 min at room temperature. Supernatant medium was decanted, and 1.0-ml samples from representative cultures were taken for radioactivity measurements. The cell pellets were resuspended in 10 ml phosphate-buffered saline [0.01 M phosphate and 0.15 M NaCl, (pH 7.0) and recentrifuged, the fluid was decanted, and the cell pellets were retained for radioactivity measurements. ^{67}Ga and ^{59}Fe radioactivities were assayed with a well-type dual-channel spectrometer with a 70- to 110-keV window for ^{67}Ga and a 600- to 1400-keV window for ^{59}Fe. Counting of separate ^{67}Ga and ^{59}Fe standards allowed the appropriate small corrections to be made to dual tracer assays for "spillover" between counting windows. In each experiment several cultures containing all of the additions except cells were included to check that sedimentable non-cell-bound radioactivity did not constitute a substantial fraction of any apparent cell-bound radioactivity measured.

Kinetics of Tracer Uptake. A large number of 5-ml cultures were set up, and additions of ^{67}Ga, ^{59}Fe, and human transferrin (in that order) were made to pairs of cultures at 48, 28, 8, 4, and 2 hr before their simultaneous harvest. They were then processed for measurement of cell-associated ^{67}Ga and ^{59}Fe as described above.

Cellular Retention of Tracers. Cells in a 100-ml culture were labeled with ^{67}Ga and ^{59}Fe during a 24-hr incubation in medium containing 10 μg human transferrin per ml. The labeled cells were then sedimented, resuspended in fresh culture medium, and divided into 5 equal parts. From 4 of these the cells were immediately sedimented and retained as references. The 5th part was diluted into fresh normal culture medium to a cell concentration that allowed another 24 hr of exponential growth. At the end of this 24-hr period, these cultures were centrifuged and counted for ^{67}Ga and ^{59}Fe activities along with the references.

RESULTS

In the following results each data point is the mean value of measurements from duplicate cultures; the average variation between duplicates is about 10%. The reproducibility of the system has also been illustrated in a previous paper (14).

Kinetics of Transferrin-promoted Uptake. Chart 1 shows the results of an experiment in which ^{67}Ga, ^{59}Fe, and human transferrin were added to HPC-108 cell cultures at various times before harvest. The cell concentrations in all cultures were identical at the time of final measurement of cell-bound radioactivity, and kinetic responses were measured for each of 4 different transferrin concentrations. The form of the experiment is such that the results reflect the rate of increase in cell concentration as well as the rate of uptake of tracers per cell. With a cell population-doubling time of 12 hr, the number of cells exposed to isotopes and transferrin during the 1st half of the 48-hr-total period is a small

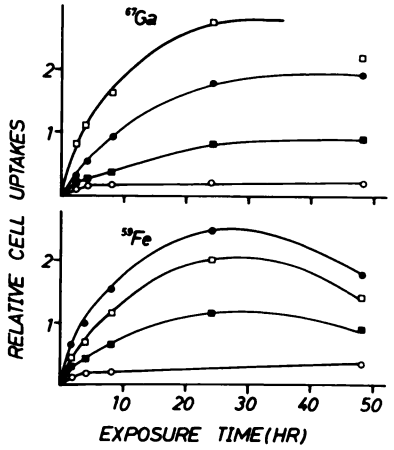

Chart 1. Kinetics of ^{67}Ga and ^{59}Fe uptake by HPC-108.1 cells. Both tracers and human transferrin were added to cultures at the indicated time prior to measurement of cell-associated radioactivity. ○, no transferrin added; ■, 2 μg transferrin per ml; ●, 10 μg transferrin per ml; □, 50 μg transferrin per ml. Each *point* represents the mean of duplicate determinations.

proportion of the final cell number, and thus one would not expect much increase for 48- over 24-hr uptakes. In several such experiments the typical ^{67}Ga result approached a plateau towards 48 hr, but for ^{59}Fe the 48-hr uptake was in fact always lower than the 24-hr uptake. Another characteristic difference between the 2 tracers (Chart 1) was that ^{67}Ga uptake increased with transferrin concentration within the range tested, whereas the ^{59}Fe response declined at high (50-μg/ml) transferrin concentrations.

Retention of Cell-associated Tracers. Cells labeled with ^{67}Ga and ^{59}Fe in the presence of transferrin were tested for tracer retention after 2 generations of cell multiplication in medium containing neither tracers nor active transferrin. Results showed that for each tracer cellular retention over this period was virtually 100%. These measurements refer to cellular binding that is sufficiently firm to withstand the usual washing procedure.

Dependence of Uptake on Transferrin Concentration. Cell-associated radioactivity was measured after 24-hr incubations of cells with the 2 tracers and with the addition of transferrin to final concentrations ranging between 0 and 200 μg/ml. Chart 2 depicts the results for both human and bovine transferrin. The titration curves are characteristically different for the 2 tracers. With increasing concentrations of human transferrin, ^{59}Fe uptake rose steeply, passed through a maximum at about 20 μg/ml, and then gradually declined, whereas ^{67}Ga uptake rose steeply toward an eventual plateau. The maximum uptakes were 10- to 20-fold above those occurring without added transferrin. Relative to tracer concentrations in the medium, maximum ^{67}Ga uptake corresponded to a 35-fold concentration, and ^{59}Fe corresponded to a 100-fold concentration of isotope by the cells. With increasing concentrations of bovine transferrin, no uptake stimulation was detectable for ^{59}Fe, but a significant and progressive stimulation of ^{67}Ga uptake did occur. The ^{67}Ga result is noteworthy in that 50 μg bovine transferrin per ml is equivalent to the transferrin concentration in less than 2% bovine serum, and yet its addition to cultures containing 10% fetal calf serum resulted in significant stimulation of ^{67}Ga uptake.

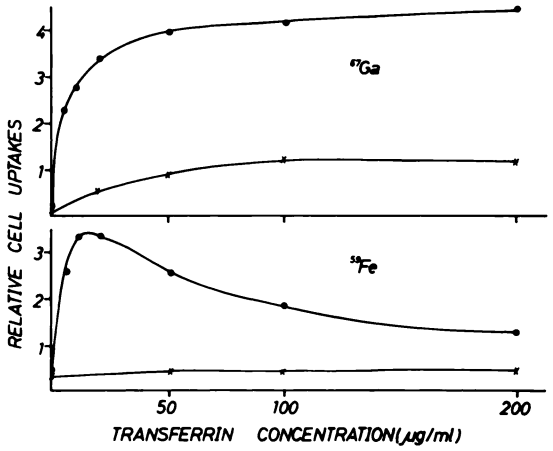

Chart 2. Dependence of 24-hr uptake of ^{67}Ga and ^{59}Fe by HPC-108.1 cells on concentrations of human (○) and bovine (×) transferrin. Final cell volume was 5.5 μl per 5-ml culture, and tracer levels in the medium were: ^{67}Ga, 387,000 cpm/ml; ^{59}Fe, 22,500 cpm/ml. Maximum ^{67}Ga cellular uptake (at 200 μg human transferrin per ml) was 69,000 cpm/culture. Maximum ^{59}Fe uptake (at 20 μg human transferrin per ml) was 11,500 cpm/culture. Each *point* represents the mean of duplicate determinations.

Effects of Added Iron. The effect of iron saturation on the ability of transferrin or serum to stimulate tracer uptake by HPC-108 cells was investigated in several ways. The 1st was to measure uptake in the presence of human transferrin that had been pretreated with various amounts of ferric citrate; the results of such an experiment are shown in Chart 3. The estimated amount of Fe^{3+} required to saturate the iron-binding capacity of transferrin is shown, and the ^{59}Fe uptake stimulation had fallen to zero at about this level as expected. In contrast, the stimulated ^{67}Ga uptake declined more gradually, the added transferrin retaining some significant activity well beyond its point of iron saturation.

The inhibition of ^{67}Ga uptake by iron was further investigated by testing the stimulatory activity of different concentrations of human serum that had been pretreated with an approximately saturating amount of Fe^{3+}. The results (Table 1) show that ^{67}Ga uptake stimulation by the larger (1.5%) serum addition was greatly reduced by iron pretreatment, whereas that by the smaller (0.25%) serum addition was much less affected by the same iron pretreatment. As in the previous experiment, an addition of about 0.02 μg Fe^{3+} caused a substantial reduction in ^{67}Ga uptake, but unlike ^{59}Fe uptake the ^{67}Ga response seemed to be affected more by the amount of iron added to the cultures than by the degree of iron saturation of the added serum or transferrin.

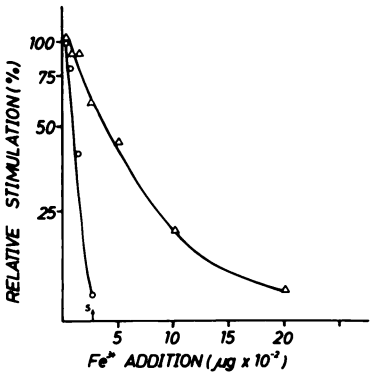

Chart 3. Effect of iron pretreatment on the ability of human transferrin (5 μg/ml) to stimulate ^{67}Ga and ^{59}Fe uptake by HPC-108.1 cells. ○, ^{59}Fe; △, ^{67}Ga; *S*, the iron concentration expected to saturate the iron-binding capacity of the transferrin. Each *point* represents the mean of duplicate determinations.

Table 1

Effect of iron loading on human serum promotion of tracer uptake by HPC-108.1 cells

Human serum addition		Cellular uptake of tracer (cpm/culture)	
Concentration (%)	Pretreatment (37°; 24 hr)	^{67}Ga	^{59}Fe
0		2,100	520
0.25	0.85% NaCl solution	9,000	2,300
0.25	Iron[a]	7,000	850
1.5	0.85% NaCl solution	15,200	1,730
1.5	Iron[a]	4,500	660

[a] Whole serum was incubated with ferric citrate at an iron concentration of 3.4 μg/ml.

DISCUSSION

Enlarging upon previous work (14), these studies have shown that both ^{67}Ga and ^{59}Fe uptakes by cultured mouse tumor cells can be greatly stimulated by the addition to the culture medium of serum or transferrin from certain species. Human transferrin gave high uptake stimulations for both ^{67}Ga and ^{59}Fe, whereas bovine transferrin was relatively inactive in each case. Transferrin and tracer additions to the system were small in relation to the amounts of bovine transferrin and iron contained in the 10% fetal calf serum component of the culture medium. The influence of 10% fetal calf serum was kept constant throughout the present experiments by the use of fetal calf serum from a single commercial batch, but its significance has to be appreciated in interpreting the results.

Kinetic studies of transferrin-promoted ^{67}Ga and ^{59}Fe uptake showed that incubation periods of at least several hr were required to obtain measurements substantially above background. Long-term kinetic experiments, however, were complicated by the continuing and rapid process of cell growth. Thus interpretations of the kinetic results in Chart 1 have to allow for exponential cell population growth and an experimental design in which the tracers and transferrin were added at various times before the final and simultaneous harvest of all the cultures. When these are taken into account, the results suggest that the rate of uptake per cell is constant for both ^{67}Ga and ^{59}Fe. The reduced 48-hr uptake of ^{59}Fe compared with the 24-hr uptake is probably due to some slow process that renders ^{59}Fe unavailable for transport into cells (perhaps exchange with nonradioactive iron on transferrin of the fetal calf serum). Our general experience has been that this effect does not appear with ^{67}Ga. From the results of kinetics experiments, it was decided to use a 20- to 24-hr incubation period in subsequent uptake measurements.

For human transferrin the dose-response relationships for both ^{67}Ga and ^{59}Fe were approximately linear at low transferrin concentrations (Chart 2). To explain their divergence at higher concentrations, we postulate the onset of transferrin saturation (*e.g.*, of cell surface receptors) in the region of 20 to 50 μg/ml. It is then suggested that the presence of excess or noninteracting transferrin can progressively reduce the proportion of ^{59}Fe available for cellular uptake. That effect is readily explained in terms of the very high transferrin affinity of ^{59}Fe (1). In the ^{67}Ga case and for the range of transferrin concentrations studied, no such reduction of uptake response would be expected because the transferrin affinity of ^{67}Ga is much lower (Ref. 4; R. G. Sephton, F. N. Cornell, and S. De Abrew, unpublished observations).

The same reasoning is tentatively applied to the bovine transferrin titrations. However, to explain the low ^{67}Ga and negligible ^{59}Fe stimulations observed, we have additionally to propose that this transferrin molecule either has a low affinity for cell surface receptors and/or is relatively inefficient in activating the cellular uptake process. It remains difficult to understand why even low ^{67}Ga responses are observed with additions that are still low in relation to the amount of fetal calf transferrin already present in the culture medium. The explanation may be forthcoming when more is known about the states of bovine transferrin and iron in the fetal calf serum.

Another possibility for analogy between ^{67}Ga and ^{59}Fe was explored by testing the uptake-promoting activity of transferrin or serum that had been pretreated with various amounts of nonradioactive iron (Chart 3). As expected, saturation of the iron-binding capacity of transferrin in this way preempted its ability to promote ^{59}Fe uptake. By contrast, it required much larger than saturating amounts of iron to reduce substantially its subsequent activity toward ^{67}Ga. The finding that ^{67}Ga uptake was reduced by addition of larger amounts of iron-loaded serum suggested that the effect of iron on ^{67}Ga uptake was dependent on the total amount of iron added to the culture system rather than on the iron-saturated state of the added transferrin. These experiments produced further evidence that, in spite of some similarities, there are substantial differences between the uptake mechanisms of these 2 tracers.

Further work is required before a detailed picture of the ^{67}Ga uptake mechanism can be derived from cell culture experiments. These results, however, indicate that mouse myeloma cells are capable of interactions with transferrin that initiate both ^{67}Ga and ^{59}Fe uptake mechanisms. Both tracers eventually become irreversibly cell bound, possibly to some intracellular component(s). However, there have also been revealed some marked differences between ^{67}Ga and ^{59}Fe uptake responses. These may possibly be explained in terms of their different transferrin affinities, or they may reflect other, more basic differences between their uptake mechanisms. Qualitatively similar findings have been gained with the range of cultured cell types referred to in our previous report (14); the preceding comments therefore apply to other mouse tumor cells, possibly to other mammalian cells as well.

One of the chief aims of this work is an eventual explanation of ^{67}Ga *in vivo* distributions. These are characterized by high uptakes in certain tumors and relatively low uptakes in hemopoietic tissues, in marked contrast with ^{59}Fe distributions. It may be that such different cell populations have in common the property of interaction with transferrin but are otherwise distinguished by different capacities for transporting intracellularly or binding the 2 tracers.

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