Liposome-Mediated Delivery of Gallium to Macrophage-Like Cells *in Vitro*: Demonstration of a Transferrin-Independent Route for Intracellular Delivery of Metal Ions

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Received October 16, 1992; accepted January 7, 1993

Gallium (Ga) prevents the activation of macrophages and might be useful as an immunosuppressive agent. It is taken up by the malignant cells through the transferrin (Tf) receptor pathway, but this pathway may be insufficient in the case of non-malignant cells. We studied the Tf-independent, liposome-mediated delivery of Ga to macrophage-like cells *in vitro* by a growth inhibition assay. The growth inhibitory properties of Ga for other types of cells was also evaluated. Ga complexed with nitrilotriacetate (GaNTA) and encapsulated in DSPG-liposomes was 16 and 48 times more potent for RAW 264 cells than free GaNTA and Ga-nitrate, respectively. CV1-P cells were also somewhat sensitive to liposomal Ga, but other cell lines with lower endocytotic capacity were insensitive. The inhibition of RAW 264 cell growth induced by liposomal or free GaNTA was partially reversed with iron-loading of the cells, indicating that this form of Ga causes an intracellular iron deficiency similar to that produced by Tf-bound Ga. Our results indicate that encapsulation of Ga in negatively charged liposomes provides a transferrin independent route for intracellular delivery of the compound to macrophages, which is of special interest in the treatment of autoimmune diseases, such as rheumatoid arthritis.

KEY WORDS: gallium; liposomes; intracellular delivery; macrophages; transferrin; *in vitro*.

INTRODUCTION

Gallium, a group IIIA metal, first used for diagnostic bone scans, has recently proved to be effective in the treatment of certain malignancies (1) as well as in the treatment of cancer-associated hypercalcemia (2). It has also been reported to prevent adjuvant-induced arthritis in rats (3). *In vitro,* gallium inhibits the proliferation of human leukemic cells (4) and T and B lymphocytes (3,5). It also prevents the activation of macrophages induced by γ-interferon (3). Because of its effects on macrophages and lymphocytes, it has been suggested that gallium may be useful as an immunosuppressive agent in the treatment of autoimmune diseases, such as rheumatoid arthritis (3,5).

Gallium enters into cells via the transferrin (Tf) receptor pathway and competes with iron for cellular uptake (6). Gallium bound to transferrin (Tf-Ga) produces an intracellular iron deficiency and suppresses cell proliferation by inhibiting the iron dependent M2 subunit of ribonucleotide reductase, thus inhibiting DNA synthesis (7). Gallium is also taken up into cells by a transferrin independent mechanism, but this mechanism accounts for only ten percent of total gallium uptake (6). In the light of clinical trials of gallium nitrate as a chemotherapeutic agent, the Tf-independent uptake of gallium may, however, be of significance (1,6). The approach of using gallium as a macrophage suppressive agent further emphasizes the significance of the Tf-independent route of gallium delivery, because Tf-receptor expression is limited in normal tissues, and nonmalignant cells show a lower expression of surface Tf receptors than their malignant counterparts (8).

Liposome-encapsulated drugs rely on the adsorptive endocytosis of the liposome for their efficient delivery to cells (9), and liposomes have proved to be effective carriers of the compounds, which otherwise would not be internalized by cells (9–11). This kind of drug has been defined as a liposome-dependent agent (10). The encapsulation of gallium in liposomes may provide a Tf-independent route for gallium delivery. This liposome-dependent delivery of gallium is especially interesting in terms of the possible use of the compound as a macrophage suppressor, since liposomes are avidly phagocytized by these cells (12).

In this study, we have examined the liposome-mediated delivery of gallium to macrophage-like RAW 264 cells *in vitro* with growth inhibition assay. The interaction of gallium with other types of cells is compared to that with macrophage-like cells. The role of iron in the mechanism of action of liposomal gallium in RAW 264 cells is also studied.

MATERIALS AND METHODS

Materials

Gallium (III) nitrate, nitrilotriacetic acid (NTA), and hematoxylin trihydrate were purchased from Aldrich Chemical Co. (Milwaukee, WI). Hemin, hexamethylene tetramine, isonicotinic acid hydrazide (isoniazid), and pyridoxal hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO). Distearoylphosphatidylglycerol (DSPG) was obtained from Avanti Polar Lipids (Birmingham, AL), and cholesterol from Sigma. They were stored ampouled in chloroform under argon at −20°C. Dulbecco's modified Eagle's medium, 10,000 U/mL penicillin and streptomycin, and fetal bovine serum were from GIBCO (Grand Island, NY). Sephadex G50 was obtained from Pharmacia (Piscataway, NJ). All other reagents were obtained from various suppliers and were reagent grade or better.

Encapsulation of Gallium in Liposomes

A stock solution of gallium (Ga) complexed with NTA (GaNTA) for liposome encapsulation was prepared by dissolving Ga-nitrate in deionized water at a concentration of 100 m*M*, and NTA was added to solution at a molar ratio (Ga:NTA) of 1:1.5. Sodium bicarbonate powder was used to

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0724-8741/93/0800-1130$07.00/0

adjust the pH to 7.2, and the solution was filter sterilized (0.2-μm syringe filter, Nalgene, Rochester, NY).

Liposomes were prepared by reverse-phase evaporation (REV) (13) from DSPG:cholesterol (67:33) and is subsequently referred to as DSPG liposomes. Twenty micromoles of DSPG and 10 μmol of cholesterol was suspended in 2 mL of diisopropylether, to which 0.8 mL of drug solution was added. The mixture was sonicated at 55°C for 10 min to produce an emulsion, and the ether was eliminated by rotary evaporation at 55°C. The liposomes were separated from unencapsulated drug by passage through a 1 × 10-cm Sephadex G50 column equilibrated with 75 m*M* NaCl, 50 m*M* morpholinoethanesulfonic acid, 50 m*M* hydroxyethylpiperazine-*N'*-2-ethanesulfonate, pH 7.0 (MES/HEPES buffer). Sterility was maintained throughout the procedure as previously described (9). The turbid liposome fractions were combined and analyzed spectrophotometrically for gallium (14) after disrupting the liposomes with 0.1% Triton X-100. The lipid content of liposomes was measured by phosphorus analysis (15). The size of these particular liposomes was not measured, but the REV procedure produces usually large uni- or oligolamellar vesicles with a mean diameter of 200–500 nm (13; Mönkkönen *et al.*, unpublished data).

Preparation of PIH and FePIH

Pyridoxal isonicotinoyl hydrazone (PIH) and ferric-PIH (FePIH) were prepared according to the method of Ponka and co-workers (16). Equimolar solutions of isoniazid and pyridoxal hydrochloride in 0.1 *M* sodium acetate buffer were heated in a boiling water bath for 5 min. The PIH precipitate, formed on cooling, was washed with water, filtered, and dried. FePIH complex was obtained by combining PIH with a solution of ferric citrate (1 mol Fe^{3+}:10 mol citrate) at a ratio of 2 mol of PIH:1 mol of Fe^{3+}. The insoluble FePIH precipitate was washed, filtered, and dried. FePIH was resuspended in MES/HEPES buffer, and the pH was lowered to 3 with 0.1 *N* HCl. A 1 m*M* stock solution of FePIH, prepared in this way, did not alter the pH of the cell culture media when added to medium to achieve a final concentration of 30 μ*M* FePIH.

Cells and Cell Growth Studies

The growth-inhibitory properties of encapsulated GaNTA, free GaNTA, and free Ga-nitrate were studied on four cell lines: a murine macrophage cell line, RAW 264, an African green monkey kidney cell line, CV1-P, a murine fibroblast cell line, L929, and a murine myeloma cell line, XCl.5/51. They were obtained from previously described sources (10,17). All cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 100 U/ml penicillin and streptomycin in a 7% CO_2 atmosphere at 37°C. Growth inhibition experiments were carried out as described by Heath and co-workers (9–11). Cells were plated at 2 × 10^4 (CV1-P), 3 × 10^4 (XCl.5/51), or 4 × 10^4 (RAW 264 and L929) cells per well in 24-well plates (Corning, NY) and allowed to grow overnight. Three wells were used to obtain the original cell count. Triplicate wells were then treated with 10 μL of drug solutions from a half-logarithmic dilution series. On each plate, three control wells were treated with 10 μL of buffer. The cells were returned to incubation at 37°C for 48 hr, except CV1-P cells, whose growth period was 72 hr because of slower growth of these cells compared to others (11). After growth, cells were counted in a Coulter Model ZM counter. XCl.5/51 cells were suspended directly in medium and the others were freed of medium and resuspended by treatment with 1 mL of phosphate-buffered saline/1 m*M* EDTA with (CV1-P) or without (RAW 264, L929) 0.05% trypsin. Percentage of cell growth was determined according to the equation

$$\% \text{ growth} = \frac{(\text{sample count} - \text{original count})}{(\text{control count} - \text{original count})} \times 100$$

The described conditions (original cell number, plate size, and growth period) allow for logarithmic cell growth over the entire period of the assay (9–11).

The cell viability test was carried out similarly to the growth inhibition assay, but instead of counting the cells at the end of the growth period, the cell viability was tested by trypan blue exclusion.

The effect of intracellular iron on the growth inhibitory properties of gallium was studied by loading RAW 264 cells with iron by hemin or FePIH. Hemin was dissolved in 0.2 *M* $Na_2CO_3/NaHCO_3$ buffer, pH 10, and maintained as a 1 m*M* (100×) stock solution. At the time of cell plating, 10 μ*M* hemin was added to each well, and the growth inhibition assay was performed as described. Hemin was omitted in control plates. At the concentration used, hemin itself did not cause an inhibition of cell growth.

In FePIH experiments, RAW 264 cells were grown in medium containing 30 μ*M* FePIH for 2 weeks (four passages). The iron-loaded cells were plated in fresh medium without FePIH, and the growth inhibition assay was conducted. With this procedure, FePIH itself did not affect the cell growth.

The effect of ammonium chloride (NH_4Cl) on the growth inhibitory properties of gallium was also studied as described earlier (10). In those experiments, a growth inhibition assay with RAW 264 cells was conducted in the presence or absence of 7.5 m*M* NH_4Cl, which was added 30 min before drug addition.

RESULTS AND DISCUSSION

The chelation of gallium with nitrilotriacetate (GaNTA) was necessary for successful encapsulation of the compound in liposomes, since free gallium ions precipitate with phospholipids. The gallium concentration of liposomes prepared as described under Materials and Methods was 5.8 ± 1.7 m*M* and the drug/lipid ratio was 0.5 ± 0.2 mol of gallium/mol of lipid (mean ± SD, $n = 3$).

In order to assure the optimal delivery of GaNTA, DSPG liposomes were chosen because our previous studies have shown that highly negatively charged liposomes are best for liposome dependent drug delivery *in vitro* (9,10).

Figure 1A shows the growth inhibitory effects of GaNTA encapsulated in DSPG liposomes (Ga/DSPG), free GaNTA, and free Ga-nitrate on RAW 264 cells *in vitro*. Ga/DSPG caused a partial inhibition already at a concentration

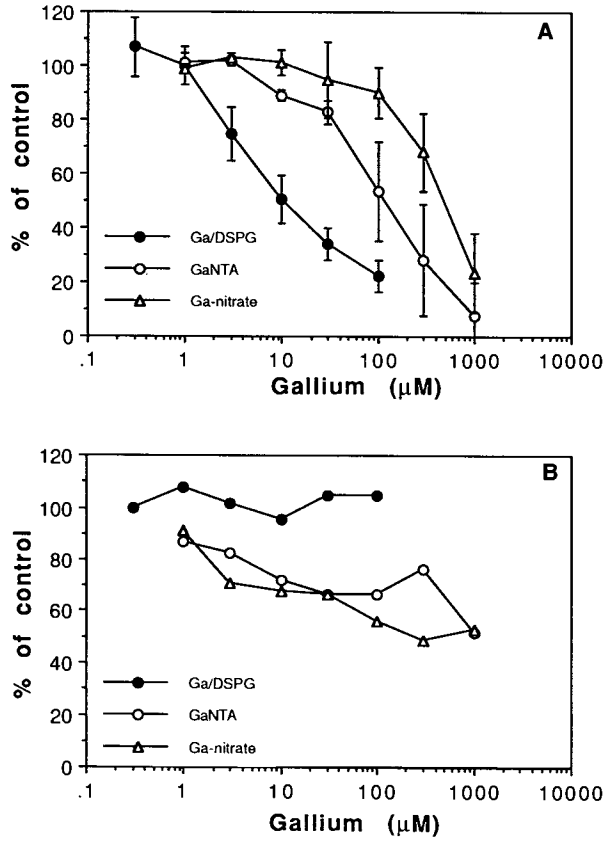

Fig. 1. The effects of GaNTA encapsulated in DSPG:cholesterol (67:33) liposomes (Ga/DSPG), free GaNTA (GaNTA), and free Ga-nitrate (Ga-nitrate) on the growth (A) and viability (B) of RAW 264 cells. Growth inhibition data represent the mean ± SD of four independently conducted experiments; viability data are from one representative experiment.

of 3 μ*M*, and at 100 μ*M* the cell growth was 22.2% of control. Because the drug/lipid ratio in Ga/DSPG liposomes was 0.5, the highest concentration of DSPG used for drug delivery was 200 μ*M*. DSPG has no effect on the growth of RAW 264 cells at concentrations up to 300 μ*M* (9), and this result was confirmed here (data not shown). Thus, the growth inhibition caused by Ga/DSPG was due to encapsulated GaNTA, not to phospholipid.

Ga/DSPG was far more potent than either form of the free drug, although free GaNTA and Ga-nitrate also showed a progressive decrease in RAW 264 cell growth with increasing concentrations of gallium (Fig. 1A). GaNTA caused a significant inhibition of cell growth at a concentration of 30 μ*M*, while 300 μ*M* Ga-nitrate was required to affect the cells. At 1000 μ*M*, almost-total inhibition of cell growth was achieved with GaNTA, whereas Ga-nitrate caused 76.6% inhibition.

Figure 1B shows the effect of different gallium preparations on the viability of RAW 264 cells. Ga/DSPG was not cytotoxic for the cells, but both forms of the free drug affected the viability of the cells considerably.

The results indicate that gallium is a liposome-dependent drug for RAW 264 cells, because it can be delivered into these cells much more effectively when encapsulated in negatively charged liposomes. Liposomal GaNTA seems to be cytostatic, not cytotoxic, while the growth inhibitory effects of free GaNTA and Ga-nitrate are, at least partially, attributable to cytotoxicity. Free GaNTA was a more potent growth inhibitor of the cells than free Ga-nitrate, suggesting a better delivery of gallium from the chelator nitrilotriacetate than from nitrate salt. About 90% of gallium from Ga-nitrate is taken up by cells through the transferrin receptor pathway (6). Macrophages can, however, take up iron from nitrilotriacetate independent of transferrin (18), and this is possible for gallium also. It seems that this chelator-mediated route is even more effective for gallium delivery in macrophage-like cells than the transferrin receptor pathway.

The growth inhibitory properties of gallium for CV1-P, L929, and XCl.5/51 cell lines are shown in Fig. 2. Liposomal GaNTA was delivered into CV1-P cells more effectively than

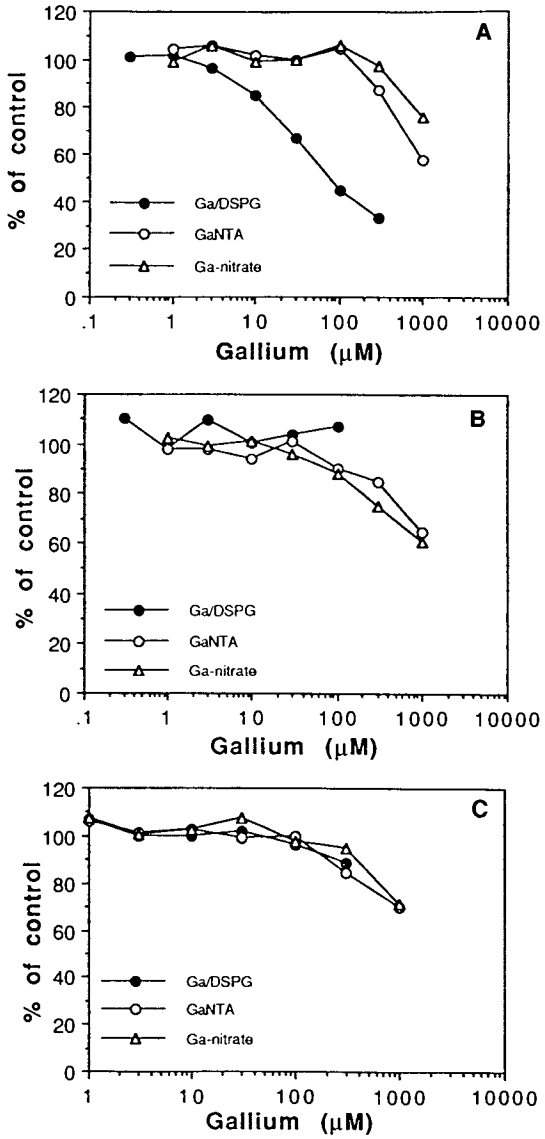

Fig. 2. The effects of GaNTA encapsulated in DSPG:cholesterol (67:33) liposomes (Ga/DSPG), free GaNTA (GaNTA), and free Ga-nitrate (Ga-nitrate) on the growth of CV1-P (A), L929 (B), and XCl.5/51 (C) cells.

either form of the free drug (Fig. 2A), whereas L929 fibroblasts (Fig. 2B) and XCl.5/51 myeloma cells (Fig. 2C) were not sensitive to Ga/DSPG. For any form of gallium, all these cell lines were far less sensitive than RAW 264 cells, XCl.5/51 showing almost-total resistance.

Based on IC_{50} values (Table I), GaNTA encapsulated in liposomes was 16 and 48 times more potent for RAW 264 cells than free GaNTA and Ga-nitrate, respectively. For CV1-P cells, the corresponding difference could not be measured, because the inhibitory effects of free drug did not reach 50% of control at the concentrations studied (Fig. 2A). For liposomal GaNTA, CV1-P cells were about seven times less sensitive than RAW 264 cells.

The results indicate that the liposome-dependent delivery of gallium is limited to the cells with a high endocytotic capacity, such as macrophage-like cells and CV1-P, which is a well-characterized cell line for liposome–cell interaction (10). The IC_{50} values of every form of gallium are at least micromolar, so gallium is lot less potent than most of the liposome-dependent drugs studied, e.g., methotrexate-γ-aspartate and *N*-(phosphonoacetyl)-L-aspartic acid (9–11). Consequently, the resistance of L929 and XCl.5/51, the cell lines with a lower endocytotic capacity, may be explained by the failure of gallium to reach sufficient intracellular drug concentration to affect the cell growth. This is supported by the finding that RAW 264 and CV1-P cells are sensitive, whereas L929 cells are insensitive for liposomal delivery of phosphonoacetic acid, whose IC_{50} values are also at high micromolar range (9).

However, if GaNTA gains access to the cells by penetration through cell membranes, not by endocytosis, the differences in cellular uptake will not explain the differences in potency of GaNTA seen between the cell types. It is possible that factors other than cellular uptake of gallium cause the different sensitivity of various cell lines. The IC_{50} value of free Ga-nitrate for RAW 264 (Table I) is about five times higher than reported for HL60 cells (4,19), and other cell lines used in this study were far less sensitive than RAW 264 cells for both of the free forms of gallium. Taetle *et al.* (20) have suggested that hematopoietic tumor cells are more sensitive than solid tumor cells to the cytotoxic effects of intracellular iron depletion, a mechanism by which gallium affects the cells (7,19). Thus, the corresponding differences in the sensitivity of cell lines studied may account for the results.

Table I. The Potency of GaNTA Encapsulated in DSPG:Chol Liposomes (Ga/DSPG), Free GaNTA, and Free Ga-Nitrate for Various Cell Lines *in Vitro*

	IC_{50} (μ*M*)[a]		
Cell line	Ga/DSPG	GaNTA	Ga-nitrate
RAW 264	10.5 ± 4.7[b]	164 ± 145[b]	506 ± 169[b]
CV1-P	77	>1000	>1000
L929	>100	>1000	>1000
XCl.5/51	>100	>1000	>1000

[a] The concentration of the drug required to produce 50% inhibition of cell growth.
[b] Mean ± SD; $n = 4$.

Since iron depletion is the mechanism of action of transferrin-bound gallium (Tf-Ga) in HL60 cells, we tested whether liposomal gallium acts in the same manner in RAW 264 cells. Iron from hemin can be made available to an intracellular free iron pool (21), and FePIH is a soluble iron complex that can deliver iron to cells for DNA synthesis independent of transferrin (16,22). The growth inhibition of HL60 cells by Tf-Ga can be completely prevented by coincubation of the cells with 10 μ*M* hemin (7), but FePIH does not have a striking protective effect (19).

Incubation of RAW 264 cells with different gallium preparations together with 10 μ*M* hemin resulted in a partial restoration of cell growth in the case of liposomal and free GaNTA (Figs. 3A and B). The growth inhibitory effects of Ga-nitrate, however, was slightly affected by hemin only at the highest concentration of gallium (1000 μ*M*) (Fig. 3C). The incubation of the cells with 30 μ*M* FePIH prior to the exposure to gallium led to results comparable to those obtained through coincubation with hemin (data not shown). This indicates that liposomal and free GaNTA causes an iron deficiency similar to that produced by Tf-Ga. The protective effect of hemin for RAW 264 cells was not as extensive as it is for HL60 cells with Tf-Ga (7), but, on the other hand, FePIH in our model was much more protective than reported for HL60 cells (19). This might be due to differences in the cellular handling of iron among different tumor cell types, as suggested by Lundberg and Chitambar (19), and RAW 264 cells may utilize iron from hemin and FePIH in a different way than HL60 cells.

It is likely that the effect of Ga-nitrate on RAW 264 is not intracellular but that it affects the cells by some nonspecific extracellular mechanism, which cannot be reversed by loading the cells with iron. This is supported by the fact that a very high concentration of Ga-nitrate is required to arrest the cell growth (Fig. 1A).

The coincubation of RAW 264 cells with gallium and 7.5 m*M* NH_4Cl slightly inhibited the effects of liposomal GaNTA (Fig. 4), but the growth inhibition caused by free GaNTA and Ga-nitrate was not affected by NH_4Cl. NH_4Cl is known to elevate endosomal and lysosomal pH and block the segregation of iron from transferrin after endocytosis (23). It has also been suggested that cells treated with Tf-Ga have lost their ability to acidify the intracellular compartment that allows iron to be released from transferrin (4). Thus, theoretically, gallium and NH_4Cl could have synergistic effects on the cell growth. Our results, however, do not support this interpretation, since NH_4Cl did not enhance the effects of gallium, but slightly inhibited the action of liposomal gallium.

The inhibition of drug delivery by NH_4Cl suggests that adsorptive endocytosis is involved in the effective delivery of the drug (10), although the effects of NH_4Cl on the liposomal drug delivery have been variable, depending on the cell line and drug studied (9–11). A possible interpretation of the present results is that liposomal GaNTA is delivered to cells by endocytosis, while free GaNTA and Ga-nitrate are not. This supports the view that GaNTA is able to penetrate through the cell membranes and that the uptake by the transferrin receptor pathway plays a minor role in its intracellular delivery. The only established pathway for the uptake of gallium as Ga-nitrate is a transferrin receptor-mediated en-

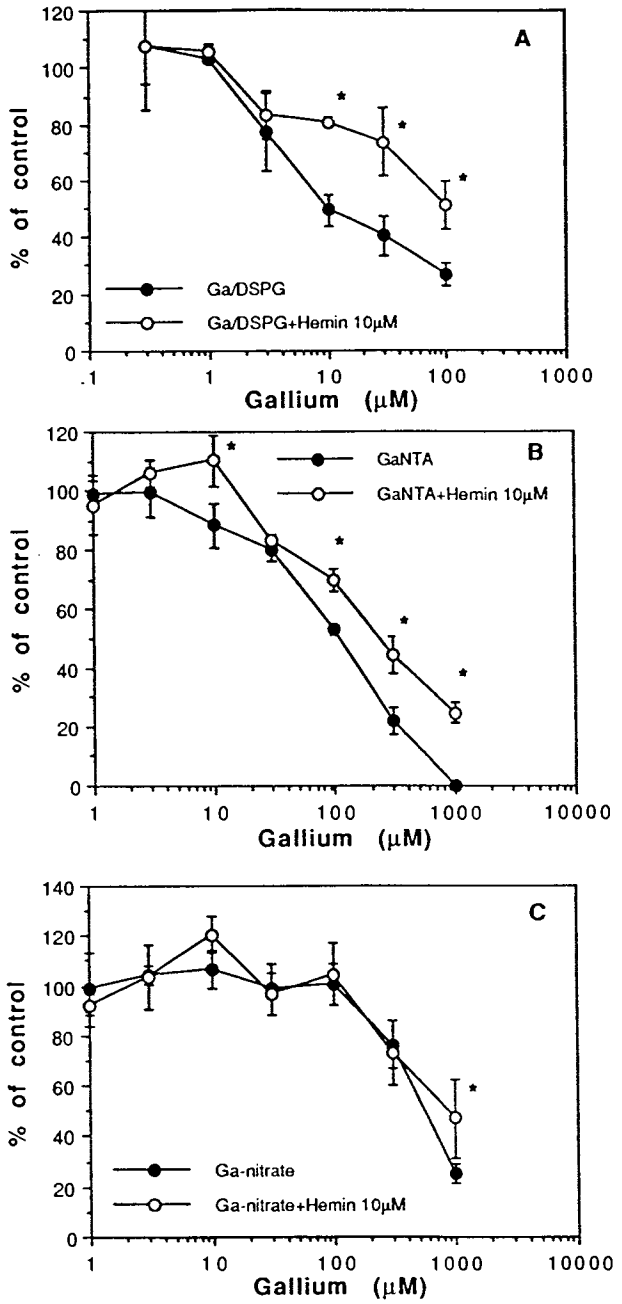

Fig. 3. The effect of hemin on the inhibition of RAW 264 cell growth by GaNTA encapsulated in DSPG:cholesterol (67:33) liposomes (A), free GaNTA (B), and free Ga-nitrate (C). Data represent the mean ± SD of three wells. (*) Significantly different from the cell growth in regular media without hemin; $P < 0.05$ (Mann–Whitney U test).

docytosis, which should have been blocked by NH_4Cl. The fact that this did not occur further supports our suggestion that Ga-nitrate in our model affects the cell growth by a nonspecific extracellular mechanism.

In conclusion, encapsulation of gallium in negatively charged liposomes provides a transferrin-independent route for intracellular delivery of the compound to macrophage-like and other cells with sufficient endocytotic capacity. This finding is of interest for possible macrophage suppression in autoimmune diseases. Especially in rheumatoid arthritis, the cessation of inflammatory processes by affecting phagocytic cells locally in the joint cavity after an intraarticular injection of gallium-liposomes might be of value, and the application of the agent for therapeutic use will depend on the *in vivo* establishment of therapeutic doses. The effects of gallium on various aspects of macrophage function, such as cytokine production, warrant further examination. Further, to our knowledge, this is the first report of the liposome-dependent delivery of metal ions. Szoka and Jones (24) delivered sodium and chromate ions as markers to cells using liposomes, to study liposome breakdown in cells. Both chromium and sodium enter cells readily, and it is unlikely that liposomes would force their delivery more effectively. Our studies with gallium demonstrate that the delivery of a metal can be promoted by liposomes and could be applied also to the delivery of other biologically active metal ions.

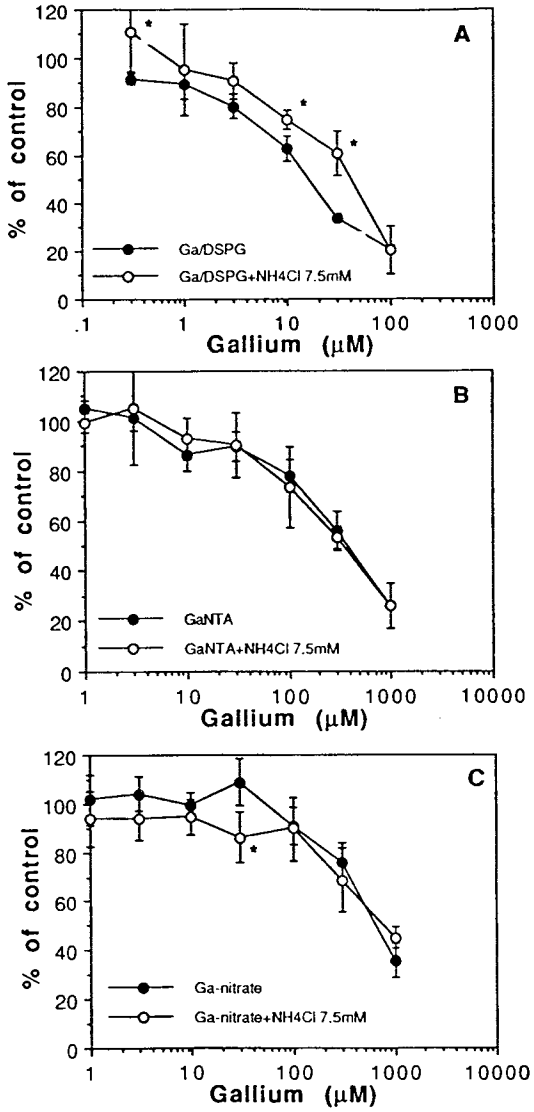

Fig. 4. The effect of NH_4Cl on the inhibition of RAW 264 cell growth by GaNTA encapsulated in DSPG:cholesterol (67:33) liposomes (A), free GaNTA (B), and free Ga-nitrate (C). Data represent the mean ± SD of three wells. (*) Significantly different from cell growth in the absence of NH_4Cl; $P < 0.05$ (Mann–Whitney U test).

ACKNOWLEDGMENT

This work was financially supported by the Technical Development Center of Finland (TEKES).

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