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In vitro drug delivery mediated by ecto-NAD$^+$-glycohydrolase ligand-targeted liposomes

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We have studied the growth-inhibitory potency of methotrexate and methotrexate-γ-aspartate encapsulated in liposomes conjugated to ligands of ecto-NAD$^+$-glycohydrolase (Salord, J. et al., Biochim. Biophys. Acta 886 (1986) 64–75). The ability of targeted liposomes to enhance growth inhibition, which amounted to a 4-fold reduction of the drug concentration required to inhibit cell growth by 50% as compared to nontargeted liposomes, was observed only with cells expressing this ecto-enzyme activity, i.e., Swiss 3T3 fibroblasts and RAJI, a Burkitt-type lymphoma cell line. Delivery of the encapsulated drugs was inhibited by NH_4Cl and varied with the endocytic capacity of the cells. Only small unilamellar vesicles affected the growth of the lymphoma cells, whereas the fibroblasts were more sensitive to large unilamellar vesicles. With vesicles of appropriate size, there was a good correlation between the specific binding of the targeted liposomes to cells and drug delivery. Our results suggest that ecto-NAD$^+$-glycohydrolase can provide a recognition site on target cells and mediate the internalization of targeted liposomes by a mechanism most probably related to adsorptive endocytosis.

Introduction

Drug delivery to specific cells by ligand-mediated targeting of liposomes represents an attractive potential in therapy [1–3]. The applicability of such an approach depends on the control of a large number of parameters such as the access of the vesicles to their target cells [4], the selectivity of the delivery system in the recognition/association step and the capacity of the targeted liposomes to enter the cells and to deliver their content to the appropriate cellular compartments, e.g., cytoplasm [1,2]. Several classes of ligands have been bound to the surface of liposomes in order to exploit this concept, among which antibodies and sugar residues have been best explored (reviewed in Ref. 1). Recently, we tested the feasibility of targeting liposomes via interaction with ecto-enzymes [5], i.e., enzymes which have their active site oriented to the external surface of the cell [6,7]. These enzymes, in analogy to other

Abbreviations: (3,4-$(CH_3)_2$)PdAD$^+$, 3,4-dimethylpyridine adenine dinucleotide; PA, L-α-phosphatidic acid; PC, L-α-phosphatidylcholine; PE, L-α-phosphatidylethanolamine; SPDP, *N*-succinimidyl-3-(2-pyridyldithio)propionate; SATA, succinimidyl-*S*-acetylthioacetate; SMPB, succinimidyl-4-(*p*-maleimidophenyl)butyrate; MPB-PE, 4-(*p*-maleimidophenyl) butyrylphosphatidylethanolamine; Hepes, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; SUV, small unilamellar vesicles; REV, large unilamellar vesicles obtained through reverse-phase evaporation.

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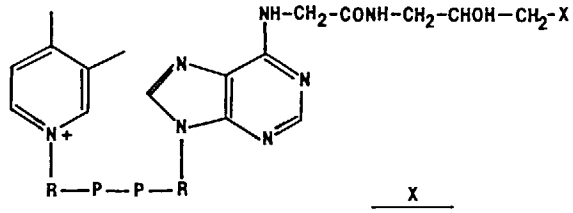
cell-surface components, can be markers of certain cell types and/or phenotypes [7–11]. We covalently linked an N^6-derivative of 3,4-dimethylpyridine adenine dinucleotide, a competitive inhibitor of ecto-NAD$^+$-glycohydrolase (EC 3.2.2.6) to preformed liposomes, and we showed that the specific binding of the conjugated liposomes to cells presenting this ecto-enzyme activity [5] was greatly increased (up to 5-fold). Since the capacity of a targeted liposome to enter a cell and to deliver its content depends notably on the cell type and on the surface molecule to which the liposome binds [12], we have now investigated the ability of ecto-NAD$^+$-glycohydrolase to mediate the uptake of liposomes by cells. Accordingly, we have studied the potency of targeted liposomes, having encapsulated methotrexate and methotrexate-γ-aspartate, to affect cell growth. Here, we present the results obtained with Swiss 3T3 fibroblasts, a model cell system which possesses an important ecto-NAD$^+$-glycohydrolase activity [5], and with Burkitt-type lymphoma cell lines for which elevated NAD$^+$-glycohydrolase activity was proposed to be a positive enzymatic marker for malignancy [13].

Materials and Methods

1,N^6-Etheno-NAD$^+$ was synthesized as described previously [14]. Deoxy[6-^{3}H]uridine (21 Ci/mmol) was from Amersham. SMPB was purchased from Pierce Chemical Co. and MPB-PE was prepared according to Martin and Papahadjopoulos [15]. SPDP and SATA were synthesized according to Carlsson et al. [16] and Duncan et al. [17], respectively. Methotrexate was a gift from Laboratoires Spécia (Paris) and methotrexate-γ-aspartate was synthesized [18] and generously provided by Dr. J.R. Piper, Southern Research Institute, Birmingham, Al. Phosphatidylcholine was extracted from egg yolk and purified according to Nielsen [19]. 5(6)-Carboxyfluorescein was obtained from Eastman Kodak Co. and was purified according to Ralston et al. [20]. Polycarbonate filters were from Nuclepore. Fluorescamine, 5,5′-dithiobis(2-nitrobenzoic acid), cholesterol, phosphatidic acid (from egg yolk PC) and phosphatidylethanolamine (egg yolk) were purchased from Sigma Chemical Co. Dulbecco's modified Eagle's medium, RPMI 1640 culture medium and fetal calf serum were obtained from Gibco and the culture flasks were from Falcon. All other reagents were of analytical grade.

Synthesis of the thiol functionalized 3,4-dimethylpyridine adenine dinucleotides. (3,4-(CH$_3$)$_2$)PdAD$^+$-N^6-[*N*-(2-hydroxy-3-aminopropyl)carbamoylmethyl] (Fig. 1; compound 1) was prepared from β-NAD$^+$ by a series of enzymatic and chemical steps as described previously [5]. This compound was then reacted with SPDP, the sulfhydryl-protective group removed by reduction with dithiothreitol and the final product (compound 2): (3,4-(CH$_3$)$_2$)PdAD$^+$-N^6-[*N*-(2-hydroxy-3-(3-thiopropionate)aminopropyl)carbamoylmethyl], was then purified by filtration on a Sephadex G-10 column [5]. In the present work, we also used an alternative functionalization method using SATA as heterobifunctional reagent [17,21]. To 150 mg (0.12 mmol) compound 1 in 170 ml of 50 mM sodium phosphate buffer (pH 7.4) was added progressively at room temperature and, under magnetic stirring, 33 mg (0.25 mmol) SATA dissolved in 1.7 ml dimethylformamide. The reaction progress was monitored by measuring the disappearance of free amino groups with fluorescamine, according to Böhlen et al. [22]. The reaction was essentially complete after 30 min and the reaction volume was then reduced under vacuum to 15 ml. The resulting compound (3,4-(CH$_3$)$_2$)PdAD$^+$-N^6-[*N*-(2-hydroxy-3-(2-*S*-acetylthioacetate)aminoprop; (compound 3) was purified by filtration on a 3 × 120 cm Sephadex G-10 column eluted with water at a flow rate of 0.5 ml/min. The pooled fractions were concentrated under vacuum to a 10



	X
1	-NH$_2$
2	-NH-CO-CH$_2$-CH$_2$-SH
3	-NH-CO-CH$_2$-S-COCH$_3$
4	-NH-CO-CH$_2$-SH

Fig. 1. Structure of (3,4-(CH$_3$)$_2$)PdAD$^+$-derived ligands.

mM concentration of compound 3. The compound, obtained in a 75% yield, was 98% pure as judged by HPLC [5]. (3,4-$(CH_3)_2$)PdAD$^+$-N^6-(*N*-(2-hydroxy-3-(2-thioacetate)aminopropyl)carbamoylmethyl], (compound 4), was obtained by treating compound 3 with a 100 molar excess of hydroxylamine in 5 mM Hepes (pH 7.4) under argon. After 30 min at room temperature, the thiol deprotection reaction was complete, as judged by sulfhydryl-group determination with 5,5′-dithiobis(2-nitrobenzoic acid) [23] and compound 4 was used immediately without any further purification.

Preparation of liposomes and coupling of the thiol functionalized 3,4-dimethylpyridine adenine dinucleotides. The molecules to be encapsulated (methotrexate, methotrexate-γ-aspartate, 5(6)-carboxyfluorescein) were in 5 mM Hepes buffer (pH 7.4) and the osmolarity of the solutions was adjusted to 290 mOsmol/kg with NaCl. Large unilamellar liposomes were prepared from 10 μmol lipids (PC, PA, MPB-PE, cholesterol at the molar ratios indicated under Results) using the reverse-phase evaporation method [24], and were extruded through 0.2 μm polycarbonate filters [25]. Small unilamellar vesicles were prepared from a suspension of 10 μmol lipids in 1 ml of Hepes buffer (pH 7.4) containing the drugs. The mixture, whose temperature was maintained at approx. 25 °C, was pulse-sonicated with a 3-mm diameter probe sonicator (Vibra Cells, Sonics and Materials Inc.) at 300 W for 30 min under a continuous flow of nitrogen. The vesicles preparation was then centrifuged at 50000 × *g* for 2 h. For the conjugation of the ligands to the vesicles, freshly prepared liposomes were mixed with a 5-fold molar excess of compounds 2 or 4 over MPB-PE content. The pH was adjusted to 6.5 and the reaction mixture was left under argon for 4 h at ambient temperature. Control liposomes (non-targeted vesicles) were similarly reacted with mercaptoethanol. Finally the conjugated liposomes were separated from reagents, unencapsulated molecules and unbound ligands by filtration on a 1 × 16 cm Sephadex G-75 column equilibrated and eluted with a 5 mM Hepes buffer (pH 7.4) containing 100 mM NaCl. The liposomes were then analyzed for their phosphorus content [26] and amount of covalently coupled ligand as described previously [5]. The concentration of encapsulated drug (methotrexate, methotrexate-γ-aspartate) was estimated by measuring the fluorescence of the absorbance [27] of co-encapsulated 5(6)-carboxyfluorescein, assuming that both drug and dye were comparatively entrapped.

Cell cultures. Swiss 3T3 fibroblasts were obtained from J.P. Beck, Strasbourg. RAJI and BJAB, Burkitt-type lymphoma cell lines were provided by G.M. Lenoir, Lyon. The fibroblasts were cultured in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum. The Burkitt's lymphoma cells were cultivated in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, glutamine (280 μg/ml) and sodium dicarbonate (2 mg/ml). The cells were grown in a 5% CO_2 atmosphere (final pH 7.4) at 37 °C.

Association of negatively charged liposomes with cells. 3T3 fibroblasts were resuspended in 3-cm diameter Petri dishes at $1 \cdot 10^5$ cells per 2 ml Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum. After an overnight adherence period, the cells were incubated for 2 h at 37 °C with 0–200 nmol liposomes composed of PC/PA/cholesterol (10 : 1.5 : 7) containing 40 mM 5(6)-carboxyfluorescein. The medium was then pipetted off and the cells were washed twice with phosphate-buffered saline. Carboxyfluorescein associated to the cells was measured fluorimetrically after cell digestion in 2 ml phosphate-buffered saline containing 0.5% (v/v) Triton X-100 [27]. A standard curve was established under the same conditions with the initial liposome preparation.

Cell growth inhibition. Fibroblasts were plated for growth inhibition at $5 \cdot 10^4$ cells per 3 cm diameter well in 2 ml of the culture medium. After 24 h of adherence at 37 °C, the cells were incubated (in duplicate), with the free or encapsulated drugs for a further 48 h. The cells were then washed carefully with phosphate-buffered saline, digested with 0.1 M NaOH (30 min) and analyzed for their protein content [28]. Growth was calculated according to the equation:

$$\% \text{ growth} = \frac{[\text{final(treated)} - \text{original}]}{[\text{final(control)} - \text{original}]} \times 100$$

Alternatively, fibroblasts and Burkitt lymphoma cells were resuspended, respectively at $5 \cdot 10^4$ and

$2 \cdot 10^5$ cells per 1 cm diameter well, in 1 ml of the appropriate growth medium. After 12 h they were treated, in duplicate, with the drugs for 4 h, followed by an addition of [^{3}H]deoxyuridine (1 μCi). Control cells were treated with the medium alone. After a 16-h labeling period, the fibroblasts were freed of the medium, washed twice with phosphate-buffered saline and detached by treatment with 0.05% trypsin (in 1 ml phosphate-buffered saline/1 mM EDTA at 37°C for 10 min). The cells were then collected on Whatman GF/B filters and rinsed with buffer followed by methanol. In the case of the lymphoma, the cell suspension was collected directly on the filters and processed. The dried filters were counted for radioactivity in 10 ml of Ready-Solv (Beckmann) scintillator.

When tested with free or encapsulated methotrexate, the two methods yielded similar IC_{50} values within a range of $\pm 15\%$. Results are given as means of duplicate determinations which did not differ by more than 10%.

Ecto-NAD$^+$-glycohydrolase activity. Intact cells were incubated at 37°C in phosphate-buffered saline (pH 7.4) in the presence of 1,N^6-etheno-NAD$^+$ (56 μM) and NAD$^+$ (550 μM). The hydrolysis of the substrate was followed fluorimetrically at 410 nm (excitation 300 nm) and the activity was calculated as described previously [14]. Nucleotide pyrophophatase activity was assessed fluorimetrically, under the same conditions, using pyridine 1,N^6-etheno-adenine dinucleotide as substrate [29]; in the cells tested, it contributed less than 10% of the observed NAD$^+$-glycohydrolase activity and was subtracted. The ecto-nature of NAD$^+$ glycohydrolase was evidenced by the lack of a rate increase of the nonpermeant substrate hydrolysis observed after the addition, to the cells, of a permeating concentration of detergent (0.1% w/v emulphogene BC 720) [14].

Results

Thiol functionalized pyridinium analogs of NAD$^+$ (compounds 2 and 4; Fig. 1), which are ligands of ecto-NAD$^+$-glycohydrolase, were covalently bound to preformed liposomes containing the maleimide derivative MPB-PE, essentially as described in our earlier publication [5]. However, in the present work the reactive thiol group was mostly introduced using SATA, a new heterobifunctional reagent [17,21]. The main advantage of SATA is that it makes possible the removal of the thiol protective group under mild conditions with hydroxylamine and direct coupling of the ligand without any further purification. Compared to other methods, this procedure proved in our case to be more convenient and less time consuming. It should be noted, however, that SATA is not without drawbacks; in other studies we found that this reagent can also act, to some extent, as an *O*- and *N*-acylating agent, but in most cases the unwanted *O*-acetyl groups were removed in the hydroxylamine step (J. Haensler, unpublished results).

Kinetics of targeted liposome interaction with fibroblasts

The association of large unilamellar vesicles bearing (3,4-(CH$_3$)$_2$)PdAD$^+$ as ligand with the ecto-NAD$^+$-glycohydrolase of 3T3 fibroblasts, was demonstrated previously by in vitro competition experiments. When high concentrations (millimolar range) of free ligand were co-incubated with

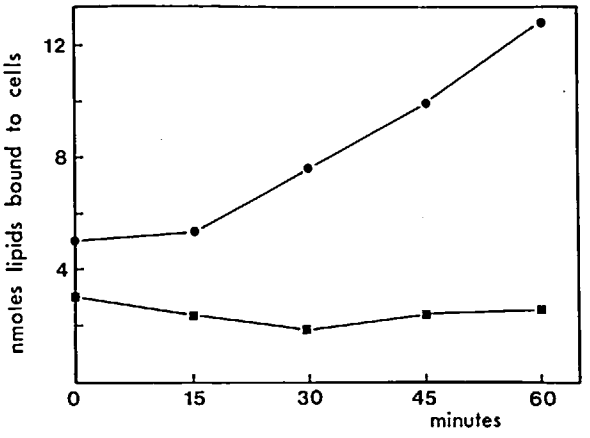


Fig. 2. Influence of the preincubation time between targeted liposomes and 3T3 fibroblasts on the inhibition of specific binding by free (3,4-(CH$_3$)$_2$)PdAD$^+$. Confluent fibroblasts ($8 \cdot 10^4$ cells in 2 ml of 5 mM Hepes/100 mM NaCl, pH 7.4) were incubated at 37°C with 200 nmol liposomes (REV) composed of PC/cholesterol/MPB-PE (9:10:1) conjugated to compound 2 and containing 50 mM 5(6)-carboxyfluorescein (●). Unconjugated liposomes (■) were composed of PC/cholesterol (1:1). At given times, 4 mM (final concentration) of (3,4-(CH$_3$)$_2$)PdAD$^+$ was added. After a further incubation of 1 h, binding was determined.

the targeted liposomes, the specific binding was largely abolished [5]. To determine whether this interaction is followed by an uptake mechanism, we pre-incubated the targeted liposomes for given times (between 0 and 60 min) followed by the addition of 4 mM of the nonpermeant (3,4-$(CH_3)_2$)PdAD$^+$ in order to displace the vesicles remaining bound to the enzyme at the surface of the cells. The results, illustrated in Fig. 2, indicate that the targeted liposomes progressively escape displacement. In contrast, the association to cells of the control vesicles remains unaffected in the presence of the free ligand. This indicates that the targeted liposomes are most probably taken up by the cells. However alternative explanations cannot be excluded, e.g., multivalent interactions between a conjugated liposome and several ecto-NAD$^+$-glycohydrolase molecules could also develop and progressively increase the magnitude of the functional affinity [5], thus rendering the displacement increasingly difficult. For that reason, we studied the effect of encapsulated drugs on cell growth.

Fibroblasts growth inhibition by methotrexate and methotrexate-γ-aspartate encapsulated in targeted liposomes

Illustrated in Fig. 3 is the effect of methotrexate, free or encapsulated in large unilamellar vesicles (REV), on the growth of 3T3 fibroblasts. When considering the concentrations of drug needed to reduce cell growth by 50% after 48 h of culture (i.e., IC_{50} values), the targeted liposomes ($IC_{50} = 0.2$ μM) were about 3 to 4-fold more efficient than controls. Both vesicle populations, however, were less effective than the free drug ($IC_{50} = 0.1$ μM). Since the significance of such results can be affected by the possible leakage of free drug from the liposomes during the growth period, we also investigated the effect of methotrexate-γ-aspartate. This molecule, while remaining a powerful inhibitor of dihydrofolate reductase, is less efficiently transported into cells [18] and its growth-inhibitory effect is liposome delivery-dependent [30]. When given free to 3T3 fibroblasts, methotrexate-γ-aspartate affected cell growth only minimally (Fig. 3) in the concentration range tested; (a 30% reduction of growth was observed at 1.6 μM; not shown). In contrast, as shown in Fig. 3, when administered encapsulated

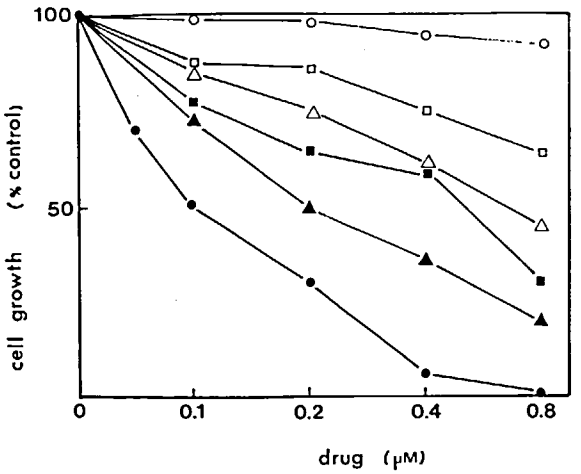


Fig. 3. Effect of free and encapsulated drugs on the growth of 3T3 fibroblasts. $6 \cdot 10^4$ cells in 2 ml growth medium were treated with increasing concentrations of free methotrexate (●), methotrexate-γ-aspartate (○) or with increasing concentrations (0–32 nmol phospholipids/ml) of liposomes (extruded REV) composed of PC/cholesterol/MPB-PE (9:10:1), conjugated to compound 4 (targeted vesicles) or mercaptoethanol (controls), containing 5 mM drugs. Methotrexate in targeted (▲) and in control (△) liposomes; methotrexate-γ-aspartate in targeted (■) and in control liposomes (□). After 48 h growth, cell protein was determined.

in liposomes (REV), the drug became active at much lower concentrations; interestingly, its potency ($IC_{50} = 0.5$ μM) was again highest in the targeted liposomes (approx. 3 to 4-fold compared to controls). This toxicity of methotrexate-γ-aspartate is a good indication for a liposome-mediated drug delivery. In control experiments we tested the action of empty liposomes of similar composition on the growth of the fibroblasts; no significant effect could observed in the lipid concentration range used (up to 200 nmol/ml).

Since the advantage of targeting was relatively modest, the effect of the size of the conjugated liposomes on their drug delivery capacity was investigated. We have studied the growth-inhibitory potency of methotrexate-γ-aspartate encapsulated in SUV, which had a mean diameter of 34 nm (determined by quasi-elastic light scattering with a Malvern Autosizer), as compared to 180 nm for the extruded REV used above. Despite the fact that the drug was encapsulated at a higher concentration, i.e., 50 mM instead of 5 mM in the REV, a concentration of drug sufficient to observe

the IC_{50} could not be reached with the small targeted vesicles: about 10% inhibition of [^{3}H]deoxyuridine incorporation was observed at 0.8 μM methotrexate-γ-aspartate, the highest concentration used with targeted SUV (not shown). It can be estimated that in order to reach a similar drug uptake level, fibroblasts should internalize, in the present case, about 15-times more targeted SUV than REV vesicles, which was not the case. One can conclude that the size of the REV vesicles does not preclude their uptake by 3T3 fibroblasts.

In order to gain a better understanding of the uptake mechanism of targeted liposomes by fibroblasts, mediated by ecto-NAD$^+$ glycohydrolase, we studied the action of ammonium ions on the growth inhibitory effect of encapsulated methotrexate. Cells which had been pre-exposed for 1 h to 5 mM NH_4Cl and treated with conjugated REV vesicles, were much less affected by the drug than the control cells; e.g., at 0.4 μM methotrexate the incorporation of [^{3}H]deoxyuridine was reduced by 10% in the NH_4Cl-treated cells compared to 45% in the untreated cells (not shown). In contrast, NH_4Cl, which in itself was nontoxic to the cells, did not affect the free methotrexate growth effect (not shown). These results suggest that endocytosis is the preferred route of uptake of the targeted liposomes and that drug leakage from the vesicles contributes only minimally to the effectiveness of encapsulated methotrexate in growth inhibition.

So far, we have only studied liposomes with a neutral lipid composition which ensures minimal nonspecific interaction with cells, and consequently promotes the specific interaction with target cells via recognition of the ecto-NAD$^+$-glycohydrolase. Negatively charged phospholipids are known to increase nonspecific interactions of liposomes with a number of cells [31]. This was also the case with 3T3 fibroblasts; e.g., incorporation of phosphatidic acid, at 13 mol%, resulted in a 3-fold increased association of the negatively charged vesicles with cells compared to the neutral controls (Fig. 4B). Therefore, in order to evaluate the efficiency of the ecto-enzyme as mediator of liposome internalization, we compared the capacity of the targeted liposomes to that of negatively charged non-targeted liposomes (REV) to deliver their content, i.e., methotrexate-γ-aspartate. We found that liposomes containing 13% PA or PG inhibited growth ([^{3}H]deoxyuridine incorporation) of 3T3 fibroblasts (Fig. 4A) as well that the conjugated liposomes (compare with Fig. 3).

Burkitt-lymphoma cells growth inhibition by methotrexate encapsulated in targeted liposomes

In an exhaustive study on human lymphoid cell lines, Skala et al. [13] have shown that NAD$^+$-gly-

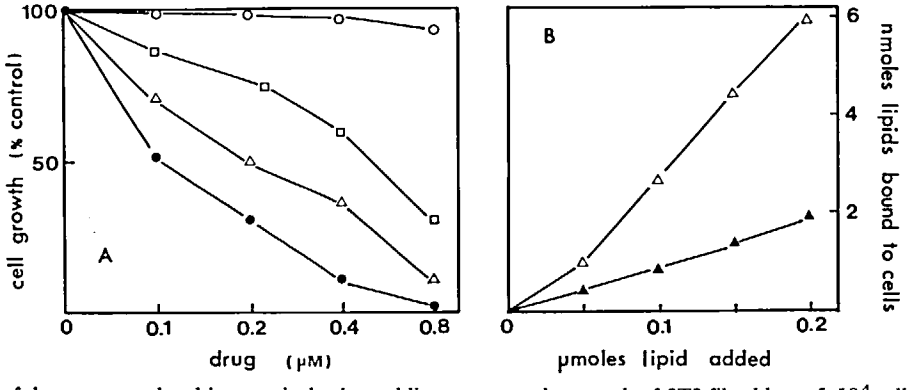


Fig. 4. (A) Effect of drugs encapsulated in negatively charged liposomes on the growth of 3T3 fibroblasts. $5 \cdot 10^4$ cells in 2 ml growth medium were incubated with increasing concentrations of free or encapsulated (5 mM) methotrexate and methotrexate-γ-aspartate. The liposomes (REV) were composed of PC/PA/cholesterol (10:1.5:7). Methotrexate free (●), encapsulated (△); methotrexate-γ-aspartate free (○), encapsulated (□). After 48 h, cell protein was determined. (B) Influence of charge on the association of liposomes to 3T3 fibroblasts. Cells were incubated for 2 h at 37°C with neutral (▲) and negatively charged (△) liposomes (REV vesicles) composed of PC/cholesterol (10:7) and PC/PA/cholesterol (10:1.5:7), respectively and containing 40 mM 5(6)-carboxyfluorescein (see Materials and Methods).

cohydrolase was highly expressed in Burkitt-type lymphoma lines [13]. However, since the high activity found by these authors was obtained from cell extracts, we first determined the cellular topology of the enzyme. Studies with intact cells revealed that, as in most other cells which are rich in NAD^+-glycohydrolase [14], the bulk of activity is associated with the outer surface of the cells. For example the RAJI line had an average ecto-NAD^+-glycohydrolase activity of 7.37 ± 1.25 $U/10^9$ cells, compared to 8.15 ± 1.85 for intact 3T3 fibroblasts. In contrast the BJAB lymphoma line, which we chose as control cells, is atypical and was practically devoid of activity. It was therefore of interest to test whether our targeted liposomes were able to deliver drugs to these cell types.

When RAJI cells were incubated with extruded REV vesicles composed of PC/cholesterol/MPB-PE (9:10:1) conjugated to compound 4, and containing 5 mM methotrexate, very little inhibition of [^{3}H]deoxyuridine incorporation was observed, e.g., about 5% at 1.6 μM drug, the highest concentration used. The free drug had an IC_{50} of approx. 0.05 μM, which is somewhat lower than that observed with the 3T3 fibroblasts (see above). This absence of effect, which could be ascribed to the size of the liposomes (see below), indicates that under our experimental conditions the vesicles used are relatively nonleaky, corroborating our earlier observations with NH_4Cl treatment (see above). When the cells were treated with SUV of similar composition, containing 50 mM drug, the targeted vesicles inhibited growth; 50% inhibition of [^{3}H]deoxyuridine incorporation being reached at 0.1 μM. The nontargeted liposomes were about 4-fold less efficient (Fig. 5). In this case also, NH_4Cl was able to protect the cells substantially against the effect of the encapsulated drug (Fig. 5). In contrast, the growth inhibitory activity was indistinguishable for targeted and nontargeted SUV in BJAB cells which lacked ecto-NAD^+-glycohydrolase. The growth inhibition curve is similar to that of nontargeted vesicles for RAJI cells (not shown).

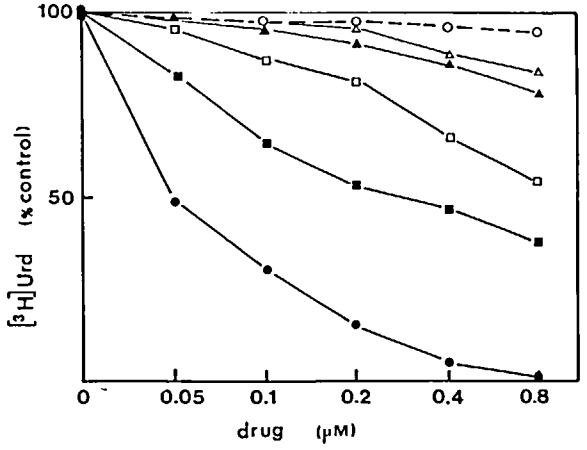


Fig. 5. Growth inhibitory effect on RAJI lymphoma cells of free or encapsulated methotrexate in large or small unilamellar vesicles. $2 \cdot 10^5$ cells in 1 ml growth medium were incubated with increasing concentrations of free methotrexate (●) or with increasing concentrations of liposomes composed of PC/cholesterol/MPB-PE (9:10:1) conjugated to compound 4 (targeted vesicles) or mercaptoethanol (controls). The vesicles contained 5 mM (extruded REV) or 50 mM methotrexate (SUV): targeted REV (○); targeted (■) and non-targeted (□) SUV. Cells pretreated 1 h with 5 mM NH_4Cl and incubated with targeted (▲) and nontargeted (△) SUV. After 20 h, the cells were collected, washed and [^{3}H]deoxyuridine incorporation determined. Control cells incorporated 58000 ± 2000 dpm.

Discussion

In a previous report we showed that liposomes conjugated to ligands of NAD^+-glycohydrolase were selectively recognized by cells which express this enzyme activity at their surface [5]. Although a prerequisite, such a ligand-mediated binding of vesicles to cells must be followed, in order to achieve drug targeting, by the internalization of the carrier and by the delivery of the drug in an active form to its cellular targets [1]. With antibody-directed liposomes, it was found previously that the internalization step depends critically on the nature of the antigen and on the target cell type [12,32]. In the present work we examined the feasibility of mediating uptake of targeted liposomes by ecto-NAD^+-glycohydrolase.

The success of the strategy using ecto-enzymes as cellular recognition sites for targeting depends on their ability to be internalized. Several ecto-enzymes are known to take part in the dynamics of cell plasma membrane; for example, in hepatocytes, 5′-nucleotidase cycles continuously between the plasma membrane and intracellular compartments [33], moreover this enzyme and other ecto-

enzymes were found to be associated with endosomes formed consecutively to receptor-mediated endocytosis [34]. The internalization of 5′-nucleotidase and alkaline phosphodiesterase was also triggered, in macrophages, by phagocytosis of latex beads, oil droplets [35,36] or *Trypanosoma cruzi* [37]. Within the present context, it is interesting that an anti-5′-nucleotidase antibody, when bound to the ecto-enzyme of rat embryo fibroblasts, follows the membrane movement and is recycled between internal structures and the cell surface [38]. Not all ecto-enzymes, however, follow the pericellular membrane flow [34], it seems that a sorting mechanism exists at the cell surface which selects the integral membrane proteins to be internalized during endocytosis [39]. At present, little is known of the factors responsible for this discrimination. We have evidence that ecto-NAD^+-glycohydrolase can be internalized, to a certain extent, during endocytosis [40]; moreover, since the conjugated liposomes present a multivalent interaction with the cells bearing ecto-NAD^+-glycohydrolase [5], the possibility exists that the formation of clusters (or micro-domains) might be a signal for its internalization [41,42].

The results obtained in this study indicate that methotrexate (or methotrexate-γ-aspartate) -containing liposomes conjugated to ligands of NAD^+-glycohydrolase present a specific growth inhibition of cells expressing this ecto-enzyme activity, i.e., Swiss 3T3 fibroblasts and RAJI, a Burkitt-lymphoma cell line. No specific association and increase in growth inhibition could be obtained with cells lacking ecto-NAD^+ glycohydrolase. The enhancement achieved with targeted vesicles was approx. 3 to 4-fold for both cell lines. Although somewhat less dramatic than the selectivities observed by other authors with antibody-targeted liposomes in the case of murine cell lines [12,30,43], our strategy gives comparable results to those obtained so far with other human lymphoma cells also using antibody-targeted vesicles [44,32]. Our data demonstrate therefore that an internalization of targeted liposomes mediated by ecto-NAD^+-glycohydrolase can be achieved.

The increases in cell growth inhibition observed with the targeted vesicles present distinct features which yield some information on the internalization mechanisms. The efficiency of the ecto-NAD^+-glycohydrolase-mediated pathway is, depending on the cell line, determined by the size of the vesicles. Large unilamellar liposomes (mean diameter 180 nm) were better for drug delivery with the highly endocytic 3T3 fibroblasts, whereas only the small unilamellar vesicles (mean diameter 34 nm) could inhibit the growth of the human lymphoma cells. Such a size discrimination depending on the cell type was already noted, particularly with lymphoma cells which so far were only found to internalize small vesicles [32,45–47]. This is probably related to the mode of internalization of the vesicles by these cells. Although direct fusion between the vesicles and the outer membrane of the cells cannot be ruled out as participating in drug delivery, it now seems evident that endocytosis is the major route of liposome uptake by cells [48]. Therefore, lymphomas, which intrinsically have a limited endocytic capacity [49], only internalize liposomes whose size can be accommodated by the coated pits/vesicles system, i.e., less than 100 nm in diameter [48]. The strong inhibition of the targeted liposomes growth effect by treatment of the target cells with NH_4Cl, favors an endocytic pathway for delivery of the encapsulated methotrexate. It is generally agreed that NH_4Cl, by raising the pH of endosomes, disrupts their function and inhibits, in the case of receptor-mediated endocytosis, the recycling of the receptors toward the cell surface and the uptake of their ligands [39]. A recycling of ecto-NAD^+-glycohydrolase between the plasma membrane and cell interior compartments could, therefore, be envisaged in order to mediate a continuous uptake of the targeted liposomes. Since a similar effect was observed with the nontargeted vesicles, we are left with the conclusion that the dynamics of the nonspecific adsorptive endocytosis is also affected by NH_4Cl. The amount of drug encapsulated by the vesicles is another determinant for optimal delivery. Only the large vesicles delivered enough methotrexate to 3T3 fibroblasts to enable observation of growth inhibition; since, apparently, these cells internalize as efficiently as the small and large vesicles, the lesser potency of the SUV might be due to the larger number of these liposomes which must be internalized in order to deliver a similar amount of drug. Interestingly, comparison of the IC_{50} values of methotrexate for the fibrob-

lasts and the RAJI cells indicates that the lymphomas efficiently endocytose SUV or (and) are more sensitive to the action of the drug.

The question remains of whether ecto-NAD^+-glycohydrolase is a good surface determinant which drives the entry of the conjugated vesicles into target cells. In principle, the fate of specifically surface-bound vesicles can be envisaged in several ways: (i) as a stable complex, which does not lead to internalization (ii) as an association which passively follows the directed plasma membrane flow (e.g., adsorptive endocytosis); in this case the specificity in drug delivery reflects that of the recognition step, and (iii) as an uptake process which can be specifically stimulated by an acceleration of the steps following initial binding (e.g., receptor-mediated endocytosis); in this case drug delivery might have a potency superior to that anticipated from binding data. In the present study, the specific enhancement of growth inhibition observed with competent targeted methotrexate-containing liposomes was strictly correlated with the specific binding of these vesicles to the target cells; e.g., for 3T3 fibroblasts, the 3- to 4-fold reduction of the IC_{50} is comparable to the increase of binding observed with the conjugated liposomes as compared to non-targeted liposomes [5]. Moreover, nontargeted negatively charged liposomes, whose charged density was adjusted to enhance nonspecific binding to a magnitude similar to the specific binding obtained by conjugation with NAD^+-glycohydrolase ligands, were as effective in reducing growth of the fibroblasts as the targeted liposomes (Fig. 4A). This indicates that ecto-NAD^+-glycohydrolase, although mediating the uptake of conjugated vesicles, does not provide a driving force superior to nonspecific endocytosis.

Altogether, these results provide evidence that ecto-NAD^+-glycohydrolase can: (i) act as a recognition site on target cells, allowing specific interaction with properly conjugated vesicles and (ii) mediate the internalization of targeted liposomes, of appropriate size, by a mechanism most probably related to adsorptive endocytosis. Since the internalization mechanisms depend on the lateral mobility of the ecto-enzymes, and on their mode of association with the cytoskeleton, it would be of interest to extend the present studies to other ecto-enzymes and possibly reach higher specificities.

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