Role of Ligand in Antibody-directed Endocytosis of Liposomes by Human T-Leukemia Cells

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ABSTRACT

The rate of uptake and intracellular processing of ligand-directed drug carriers may depend heavily on the endocytic pathway of the target antigen. We examined the role of the target antigen and type of antibody-liposome linkage in determining endocytosis of liposomes by three human T-cell leukemias, Jurkat, CEM, and Molt-4. Liposome-cell binding and internalization over time were studied using two independent assays for intracellular delivery of liposome contents: a new fluorescence assay using a pH-sensitive fluorescent dye; and a growth inhibition assay for delivery of a cytotoxic drug, methotrexate-γ-aspartate. Liposomes targeted against the transferrin receptor showed greater surface binding, internalization, and growth inhibition than liposomes targeted against the T-cell surface antigens, CD2, CD3, or CD5. Furthermore, liposomes made by conjugating the targeting antibody directly to the liposome surface were more efficiently internalized and retained than were liposomes linked to antibody-coated cells via Protein A. Selection of the type of antibody-liposome conjugate as well as the appropriate surface receptor to facilitate endocytosis is essential in antibody-directed drug treatment of cancer.

INTRODUCTION

The particular cell surface antigen which provides the target for antibody-directed cytotoxic therapy is of prime importance in determining the subsequent fate of the immunoconjugate after it binds to the cell. It is likely that the rate of antigen internalization and recycling is a vital factor in the uptake of the cytotoxic agent and its delivery to the compartment in which it exerts its toxic effect.

Recent research suggests that uptake of liposomes by cells depends on endocytosis via coated pits, followed by intracellular processing, similar to that of a number of macromolecules (1, 2). We and others have utilized this fact to design therapy of low nonspecific toxicity by encapsulating a drug or toxin which is relatively impermeant on its own and therefore dependent on endocytosis of the liposome for uptake (3–11). We have shown specific enhancement of growth-inhibitory effect of one such agent, methotrexate-γ-aspartate, when encapsulated in antibody-targeted liposomes, both with direct antibody-liposome conjugates (3, 4), and with the indirect combination of antibody-coated tumor cells with Staphylococcus aureus Protein A liposomes (5). Liposomes conjugated to Protein A proved to be 100-fold more effective than unencapsulated drug for growth inhibition of a murine T-cell leukemia, AKR/J SL2, when cells were coated with specific antibody, and 10-fold more effective than the liposomes using a nonspecific antibody (5). However, when this system was extended to human leukemia lines (12), the Protein A liposomes in combination with anti-T-cell antibodies proved to be relatively ineffective. The Protein A liposomes were also ineffective with antidiotype antibodies against a B-cell lymphoma, exhibiting specific cell association but no internalization. It was only when an anti-Dr antibody was used with the B-cell lymphoma that the Protein A liposomes had a growth-inhibitory effect greater than that with nonspecific antibody.

Because of the variability of liposome efficacy among the human cell lines, we have further explored the importance of both the type of liposome conjugate (Protein A versus direct antibody-liposome) and the role of the target antigen in promoting endocytosis of liposomes. The transferrin receptor was selected as a possible mediator of liposome uptake because of its well-defined fate in the endocytic pathway. Transferrin that is bound to its receptor is internalized via a coated-pit pathway (13, 14). Dissociation of iron from transferrin occurs in the acidic endosomal compartment (15–17), and apotransferrin and its receptor are recycled to the cell surface, where the ligand is released. Recycling thus allows the receptor to undergo further cycles of endocytosis. The continuous endocytosis and recycling of the transferrin receptor (18, 19) may make it an efficient means of endocytosis of liposomes bearing transferrin or antibodies to the transferrin receptor. Preliminary studies suggest the utility of these receptors in targeted therapy. There are reports of conjugation of transferrin itself to liposomes, resulting in successful uptake by cells (20, 21), and other reports of transferrin or antitransferrin receptor antibody conjugated to toxins enhancing uptake by tumor cells (22, 23). In the studies reported here, we have compared the uptake of liposomes mediated by antibodies against the transferrin receptor to the uptake via T-cell-specific surface antigens.

Two independent assays of endocytosis were used in these studies: a recently developed fluorescence assay using pyranine or HPTS, a pH-sensitive fluorescent dye (24, 25), as a marker of entry into the endosomal compartment (26, 27); and the other a growth inhibition assay for efficacy of methotrexate-γ-aspartate, a drug which is relatively impermeant on its own, and therefore dependent on liposome uptake to enter the cytoplasm (3).

We have also studied the effect of varying the type of antibody-liposome conjugate and type of cell receptor target on liposome endocytosis using these two assays. Protein A-liposomes with antibody-coated cells were compared to direct antibody-liposome conjugates.

MATERIALS AND METHODS

Phosphatidylcholine from egg yolk was obtained from Avanti (Birmingham, AL) and used without further purification. Cholesterol was

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5 The abbreviations used are: HPTS, 8-hydroxyxpyrene-1,3,6-pyrenesulfo

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recrystallized 4 times from cold methanol. The 4-(p-maleimidophenyl)butyrylphosphatidylethanolamine was synthesized and purified as described (28). All lipids were stored at \(-70^\circ C\) in chloroform solution under argon in sealed ampuls until use. Methotrexate-\(\gamma\)-aspartate was synthesized and kindly provided by Dr. J. R. Piper, Southern Research Institute, Birmingham, AL (29). Pyranine (HPTS) was purchased from Molecular Probes (Eugene, OR) and \(S. aureus\) Protein A from Sigma (St. Louis, MO).

Monoclonal Antibodies. Monoclonal antibodies were prepared and purified as previously described (3). Hydridoma SS.1 (anti-sheep erythrocyte, irrelevant control) was supplied by Dr. M. Coln of the Salk Institute, San Diego, CA. The remainder of the antibodies were generously supplied already purified. Leu 1 (anti-CD5) (30) was provided by Dr. T. Reichert, Becton Dickinson, Mountain View, CA; B3/25 (anti-transferrin receptor) (31, 32) by Dr. I. Trowbridge of the Salk Institute, San Diego, CA; and OKT3 (anti-CD3) (33), OKT9 (antitransferrin receptor) (34), and OKT11 (anti-CD2) (35) by Dr. G. Goldstein, Ortho Pharmaceutical, Raritan, NJ; and 14.2a (anti-GD2, irrelevant control) by Dr. R. Seeger, UCLA, Los Angeles, CA. All antibodies are of the IgG2 subclass and reactive with Protein A, except the antitransferrin receptor antibodies, which are IgG1.

Cell Lines. The human T-cell leukemia lines used for this investigation were Jurkat, CEM (36), and Molt-4 (37), kindly provided by Dr. M. Cowan at the University of California, San Francisco, and were grown in RPMI medium supplemented with 2 mM glutamine, 10% fetal bovine serum, and antibiotics in the presence of 3% CO\(_2\).

Liposome Preparation. A mixture of phosphatidylcholine, cholesterol, and 4-(p-maleimidophenyl)butyrylphosphatidylethanolamine (10:5:1 molar ratio) was suspended in a solution of 50 mM methotrexate-\(\gamma\)-aspartate or 50 mM HPTS in isotonic buffer containing 2-(\(N\)-morpholino)ethanesulfonic acid and 2-(\(N\)-morpholino)propanesulfonic acid, pH 6.7. The liposomes were then prepared by reverse-phase evaporation as previously described (3). The liposomes were extruded (38) 5 times through two 0.08-µm-pore polycarbonate membranes under sterile conditions using a high-pressure extruder (Lipex Biomembranes, Inc., Vancouver, Canada). The REV were then conjugated for 18 h at 25°C with thiolated SPA (39) or Ab (40). The conjugated REV were removed from unbound protein by flotation on a metrizamide gradient (3) with modifications previously described (5). Liposomes were then passed over a Sephadex G50 (Pharmacia) column to remove any unencapsulated drug and metrizamide, concentrated to 5 mM lipid in an Amicon YM-10 filter, extensively dialyzed against 1 M sucrose, and sterilized by passage through a 0.2-µm filter. Liposome-cell association fluorescence assay was done as above.

Fluorescent Microscopy and Fluorimetry. Fluorescence microscopy was used for visual evaluation of cell association and endocytosis. The pH-independent fluorescence of HPTS can be viewed by exciting with wide band violet (350 to 410 nm) provided by the Hoescht filter cube, reflected through long wave pass dichroic mirror (455 nm) and long wave pass barrier filter (445 nm). The second filter cube, FITC, selects narrow blue band (450 to 490 nm) for excitation with long pass dichroic mirror (510 nm) and long pass barrier filter (515 nm), such that only the fluorescence of HPTS at neutral or basic pH can be seen. The appearance of bright punctate fluorescence seen with the Hoescht cube but not with the FITC cube reflects HPTS in the low pH compartment (Ref. 27; Footnote 5). Photographs of identical fields were taken with phase contrast, Hoescht, and FITC at each time point.

Kinetic measurements of total fluorescence emission at 510 nm with excitation at the desired wavelengths were made on a SPEX Fluorolog 2 equipped with a photon counting device. Sample temperature was controlled with a circulating water bath at 20°C. Leukemia cells (2 × 10^6) were suspended in phosphate-buffered saline (2 ml) and gently mixed with a magnetic stir bar in the cuvet. The samples were excited at 403 nm (HPTS at low pH), 413 (total HPTS, invariant with pH), and 450 (HPTS at neutral or basic pH), and the emission spectra were recorded. Because endosomal and lysosomal compartments were acidic, the measured 450/413 ratio will lie between the ratio at pH 7.4 (2.0) and at pH 6 (0.2). Therefore, the fraction of HPTS-containing liposomes within the endosomal (low pH) compartment can be calculated from the formula

\[
\frac{\text{Fraction at low pH}}{\text{Fraction at neutral pH}} = \frac{R_{\text{measured}} - R_{\text{pH,7.4}}}{R_{\text{low pH}} - R_{\text{pH,7.4}}}
\]

where \(R\) is the ratio of fluorescence emission at 510 nm after excitation at 450 nm compared to excitation at 413 nm. For each liposome preparation, a 1-nmol aliquot was used as a fluorescence standard to allow calculation of the amount of cell-associated liposomes. In addition, cell autofluorescence was subtracted. The value for \(R_{\text{pH,7.4}}\) was obtained by incubation of cells with free 1 mM HPTS for 24 h, washing, and then measuring the 450/413 emission ratio. The value for \(R_{\text{pH,7.4}}\) was obtained from a similar procedure, but measuring the 450/413 emission ratio after cell lysis with 0.1% Triton X-100, so that all HPTS would be at neutral pH. \(R_{\text{pH,4.7}}\) was consistently 2.0 for all cell lines tested, corresponding to pH 7.4; \(R_{\text{pH,4.7}}\) varied with each line as follows: Jurkat, 0.6 to 0.7; CEM, 0.3 to 0.5; Molt-4, 0.5. Using the known pH dependence of HPTS emission ratios, this corresponds to an average pH of the endosomal compartment for Jurkat of 6.7; CEM, 6.4; Molt-4, 6.5. Details of the HPTS fluorimetry and fluorescent microscopy techniques, along with results validating this assay to document endocytosis in living cells, are reported by Straubinger et al. (4).

Growth Inhibition. Leukemia cells were plated at 10^4 per well in 24-well Costar plates, 1 ml/well. When SPA-liposomes were tested, the cells were pretreated with the relevant antibody at 0°C for 30 min. They were treated in triplicate with the desired concentration of drug or liposomes and then counted with a Model Fn Coulter Counter after 72-h incubation. The following calculation was made.

\[
\text{IC}_{50} = \text{determined from a plot of the percentage of growth versus log}_{\text{d}} \text{drug concentration.}
\]

RESULTS

Effect of Ligand on Endocytosis of Liposomes: Fluorescent Microscopy. Three human T-cell leukemia lines, CEM, Molt-4, and Jurkat, were studied with either soluble antibody in

\[^{4}\text{R. M. Straubinger, D. Papahadjopoulos, and K. Hong. Kinetic analysis of endocytosis and intracellular fate of liposomes using pyranine(S-hydroxy-1,3,6-pyrenetrisulfonate) as a probe, submitted for publication.}\]
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combination with Protein A-bearing liposomes, or with the direct antibody-liposome conjugate, using anti-T-surface antigen or antitransferrin receptor antibodies for targeting. Photomicrographs of identical fields were taken under phase-contrast illumination and then under fluorescent illumination with the 350- to 410-nm and 450- to 490-nm filter in order to evaluate endocytosis of the HPTS-containing liposomes at Time 0, 0.5, 1, 2, 4, and 24 h. The appearance of bright punctate fluorescence seen with the 350- to 410-nm filter but not with the 450- to 490-nm filter reflects HPTS in the low pH compartment (27, 42). Since the three different leukemia cell lines gave parallel results, detailed results of microscopy are shown only for the Jurkat cell line (Figs. 1 and 2).

A marked difference was apparent in the total cell-associated fluorescence when the indirect targeting system of specific anti-T-cell antibodies plus liposomes bearing Protein A was compared to the direct conjugates of the same antibodies to the liposome surface (Fig. 1). The SPA-REV resulted in much lower total cell-associated fluorescence after 1 to 4 h of incubation, although initial binding in the cold was equivalent to the direct conjugates. Anti-CD5 (Leu1) in combination with the SPA-REV showed a pattern consistent with capping and shedding of the fluorescent liposomes. At the end of an incubation period at 0°C, the liposomes were distributed uniformly over the cell surface. After 0.5 h of incubation at 37°C, surface fluorescence had clustered at one pole of the cell; by 4 h, considerable loss of fluorescence occurred (Fig. 1); and by 24 h, no cell-associated fluorescence was detectable. By contrast, the direct anti-CD5-liposome conjugate resulted in a higher degree of retention of cellular fluorescence. Substantial endocytosis of liposomes bearing the anti-CD5 occurred, evidenced by the pattern of HPTS fluorescence (Fig. 1). The leukemia cells with anti-CD2 (OKT11) and anti-CD3 (OKT3) also gave lower fluorescence with SPA-liposomes than with antibody-liposomes after a 4-h incubation (Fig. 1). Although endocytosis occurred with anti-CD2 and anti-CD3 antibodies with both

![Fig. 1. Cell association and endocytosis of antibody-targeted liposomes with Jurkat T-cell leukemia. Cells (2 x 10⁶) were incubated at 0°C with the relevant antibody when appropriate and then with HPTS-containing liposomes (20 nmol) conjugated to antibody or Protein A at 0°C for 45 min. Unbound liposomes were washed off, and the cells were incubated for an additional 4-h period at 37°C. HPTS fluorescence was recorded with the Hoechst filter cube (total HPTS, 350 to 410 nm). Since total fluorescence was often too faint to photograph with the FITC cube (450 to 490), particularly with the SPA-liposomes, these photos are omitted.](image-url)

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Fig. 2. Cell association and endocytosis of antitransferrin receptor-targeted liposomes with Jurkat T-cell leukemia. Cells were incubated with HPTS-containing liposomes and photographed at the indicated times as in Fig. 1. HPTS fluorescence was recorded with the Hoechst filter cube (total HPTS, 350 to 410 nm) and the FITC filter cube (HPTS at neutral pH, 450 to 490 nm). Internalization is shown by a punctate pattern evident at 350 to 410 nm but diminished at 450 to 490 nm.

direct and indirect liposomes, as shown by the appearance of bright spots seen with the 350- to 410-nm filter that disappeared with the 450- to 490-nm filter, the degree of cell-associated fluorescence was much less with the SPA-REV and was undetectable by 24 h (not shown). The antitransferrin receptor antibody was not examined with SPA-REV, since it is an IgG1 and does not bind well to Protein A.

The target antigen also had a significant effect on association and endocytosis of the Ab-REV. Only the antitransferrin receptor-bearing liposomes stained 100% of cells. The antitransferrin receptor (B3/25 or OKT9) liposomes and the anti-CD2 (OKT11) liposomes resulted in very bright total fluorescence by microscopy as well as in the greatest degree of endocytosis with complete internalization by 24 h (Fig. 2). Both anti-CD2 and anti-CD3 liposomes showed rapid endocytosis and little surface fluorescence, with a punctate pattern evident by 0.5 h and prominent by 4 h (Fig. 1). Overall uptake was less with anti-CD3 than with the antitransferrin receptor-conjugated liposomes. The anti-CD5-REV showed some capping and slower endocytosis as judged by the later development of punctate fluorescence brighter with the 350- to 410-nm filter, with lower overall cell-associated fluorescence than anti-CD2 or antitransferrin receptor-liposomes. The control (irrelevant antibody; SS.1) showed no cell-associated fluorescence with any leukemia line, as expected.

Anti-CD2 and anti-CD3 did not mediate binding of liposomes to CEM cells, nor did anti-CD3 with Molt-4, since these cell lines exhibit little reactivity with these antibodies when tested by indirect immunofluorescence by ourselves and others (40).

Cell-Liposome Binding and Intracellular Delivery by Fluorimetric Measurements. The total cell-associated liposomes and the proportion of those taken into a low pH compartment following endocytosis were evaluated by measurement of fluorescence emission at 510 nm with excitation wavelengths of 413 and 450 nm. The change in 450/413 reflects the change in mean pH to which the liposomes are exposed. HPTS fluorescence with excitation at 450 nm is pH dependent, while fluorescence with excitation at 413 nm is not. The relative endocytosis of the liposomes over time can be estimated by the decrease in the 450/413 ratio. This ratio will decrease as liposomes containing HPTS leave the neutral extracellular compartment and enter the low pH compartment of the endocytic vesicle. Fig. 3A shows the more rapid and complete endocytosis of liposomes targeted against the transferrin recep-

Fig. 3. Change in 450/413 ratio of HPTS-containing antibody-targeted liposomes by human T-leukemia Jurkat cells. Progressive uptake of liposomes into the low pH compartment of the cells is shown by the decrease in the 450/413 excitation ratio of liposome-encapsulated HPTS measured on the fluorimeter. HPTS-containing liposomes (20 nmol) conjugated to antibody (Ab-REV) or Protein A (SPA-REV) were incubated with 2 x 10^6 leukemia cells (pretreated with relevant antibody where appropriate) at 0°C for 45 min. Unbound liposomes were washed off, and the cells were incubated at 37°C for periods of 0.5, 2, and 4 h before measuring fluorescence emission at 510 nm with excitation at 413 and 450 nm. A, Jurkat cells with anti-CD5 (Leu 1) + SPA-REV ( ), anti-CD5-REV ( ), anti-CD3(OKT3)-REV ( ), and anti-CD3(OKT3)-REV ( ). B, Jurkat cells with anti-CD3(OKT3)-REV ( ), anti-CD3 + SPA-REV ( ), anti-CD2(OKT11)-REV ( ), and anti-CD2 + SPA-REV ( ).
tor compared to anti-CD5 with either SPA-REV or Ab-REV. The anti-CD5 direct and indirect liposomes have a similar average pH after 2 h. The superiority of the direct anti-CD5 conjugate in total amount of liposomes internalized as assessed by low pH fluorescence (Fig. 4) and by more effective growth inhibition (Table 1) therefore must be due to more efficient binding and retention of the liposomes at the cell surface. The reason for the difference in liposome retention may be the rapid capping and shedding observed to occur by microscopy with the indirect Protein A conjugate (Fig. 1). Fig. 3B shows the significant degree of internalization of the anti-CD2 anti-CD3-REV with the Jurkat cells. Again, little difference is seen in average pH of the direct and indirect liposomes, emphasizing the need to look at total fluorescence as well as relative acidification. Fig. 4 and Table 1 show that a greater amount of directly conjugated anti-CD2 and CD3 liposomes is internalized than the Protein A conjugate, similar to the case of the anti-CD5. The kinetic 450/413 assays of internalization of antitransferrin receptor and anti-CD5 liposomes for the CEM and Molt-4 cells were comparable to those with the Jurkat, so the results are not shown here, although the 450/413 ratios were used to calculate results for Fig. 4.

While Fig. 3 depicts the change in the 450/413 ratio over time, the measurement of both total cell-associated fluorescence and low pH fluorescence is necessary for any useful correlation with total drug delivery to the cells. The total cell-associated liposomes (as assayed by fluorescence at excitation of 413 nm) compared to the liposomes at low pH for the various target antibodies are shown at the end of a 4-h incubation at 37°C (Fig. 4). These fluorimetry results parallel the observations by microscopy using the same experimental format. The total cell-associated fluorescence and the fluorescence from the acidified liposomes were both greater with Ab-REV than with SPA-REV. The order of efficacy of the respective antibodies for internalization by Jurkat cells at 4 h is anti-CD2 > antitransferrin receptor > anti-CD5 > anti-CD3. Although the total cell-associated liposomes were approximately equal for the anti-CD5 and the antitransferrin receptor conjugates, the latter mediated enhanced liposome endocytosis. It is also important to note that the two liposome preparations bearing different antitransferrin receptor monoclonals, B3/25 and OKT9, were not equivalent. B3/25 liposomes were consistently more effective in promoting endocytosis, suggesting that minor differences between antibodies against the same target may also affect behavior of the immunoliposome conjugates. Antitransferrin receptor conjugates were both bound and internalized to a higher degree than the anti-CD5 liposomes with the CEM and Molt-4 cells.

Liposome association and uptake were also compared after a prolonged exposure (Fig. 5), under conditions simulating those used in the growth inhibition assays described below. The targeted liposomes were incubated continuously with replicating leukemia cells at two liposome concentrations, 2.5 μM and 25 μM lipid. The lower concentration corresponds to the lipid concentration at the IC50 for growth inhibition, while the higher concentration corresponds to that used in the 4-h incubation (Fig. 4). Total cell association and internalization were greatest for the antitransferrin receptor-targeted liposomes for all cell lines at both liposome concentrations. For the Jurkat cells, the antitransferrin receptor (B3/25) and anti-CD3 (OKT3) show complete internalization of cell-associated liposomes by 72 h, unlike the anti-CD5 (Leu 1) and anti-CD2 (OKT11), with which only 45 to 76% are in the low pH compartment. The proportion of internalized liposomes is higher for the B3/25 antitransferrin receptor liposomes than for the OKT9 liposomes, perhaps due to the smaller diameter of the B3/25-REV (98 nm compared to 250 nm) or to some difference in the antibody affinity. Increasing the liposome concentration 10-fold (25 μM), so that it was comparable to that in the 4-h cell association experi-

![Image](cancerres.aacrjournals.org)
containing SPA-REV was no more effective against any of the fluorescent probe. The anti-CD5 (Leu 1) with the drug-receptor > anti-CD5 and anti-CD3 > anti-CD2 (Table 1). The somes with the Jurkat cells was the same as that in the pro
sisted more effective as a direct antibody-liposome conjugate than the anti-CD5 (Leu 1).

The relative potency of the various antibody-targeted liposomes with the Jurkat cells was the same as that in the pro-longed 72-h incubation fluorimetry assay, with antitransferrin receptor > anti-CD5 and anti-CD3 > anti-CD2 (Table 1). The direct conjugates proved more effective than the SPA-liposomes for intracellular delivery (Table 1), as previously shown using the fluorescent probe. The anti-CD5 (Leu 1) with the drug-containing SPA-REV was no more effective against any of the three cell lines than an irrelevant antibody (SS.1). Both anti-CD2 and anti-CD3 antibody had greater growth-inhibitory potency as direct liposome conjugates than with the indirect Protein A liposomes with the Jurkat and Molt-4 cells. The CEM lines expressed too little of these antigens for a meaningful comparison.

A dual antibody conjugate was also tested, using drug-containing liposomes conjugated to both anti-CD5 (Leu 1) and antitransferrin receptor (B3/25), the two antibodies providing the most potent Ab-REV as well as a combination of high internalization (antitransferrin receptor) and T-cell specificity (anti-CD5). The growth-inhibitory efficacy was not significantly different from that of liposomes conjugated to either antibody alone (Table 1).

**DISCUSSION**

Two independent markers for endocytosis of liposomes were used in these studies: HPTS, a pH-sensitive fluorescent dye (25-27); and methotrexate-γ-aspartate, a growth-inhibitory drug dependent on liposome uptake for maximal potency (3). HPTS has high water solubility, low leakage, a pK< sub>9</sub> in the physiological 6–8 range, minimum interference from biological fluids due to maximum emission at 510 nm, and well-separated excitation spectra at neutral and low pH. Excitation at 413 nm results in a pH-independent emission that allows total fluorescence to be quantitated (26). Studies of encapsulated HPTS in CV-1 cells using microscopy and fluorimetry document persistence of HPTS in acidic vesicles, labeling of intracellular organelles with an acidic lumen, and reversibility of fluorescence emission with NH<sub>4</sub>Cl or monensin, which collapse cellular H<sup>+</sup> gradients. This dye was encapsulated in the liposomes to monitor their shift from the neutral extracellular compartment to the low pH of the endosome and lysosome. An aqueous phase rather than lipid phase marker was used in order to correspond to the aqueous phase cytotoxic drug.

The second assay depends on cytoplasmic delivery of the liposome-dependent drug, methotrexate-γ-aspartate. Methotrexate-γ-aspartate is a pteridine antifolate which is indistinguishable from methotrexate in its ability to inhibit dihydrofolate reductase, but 200-fold less toxic for L1210 cells than methotrexate because its influx K<sub>c</sub> is at least 100 times greater (29). However, it is equipotent to methotrexate if delivered intracytoplasmically, thus providing an indicator of liposome uptake.

The results demonstrate the importance of both the type of liposome-antibody linkage (direct conjugation to antibody or indirect association via Protein A conjugation to liposomes) and of the particular target receptor in determining liposome fate. The Protein A-bearing liposomes attached to cells pre-treated with specific antibody generally showed much lower total endocytosis than the equivalent direct antibody-liposome conjugates by both HPTS internalization and growth inhibition assay. This result is in contrast to the findings with the murine T-leukemia, AKR/J SL2, where SPA-liposomes were 10-fold more effectively taken up than the direct anti-Thy 1.1 (19E12)-liposome conjugate (5). The disparity of these findings may be due to either differences between human and murine cells or to some inherent difference in the Thy 1.1 from the CD2, CD3, and CD5 antigens. Previous studies using a human B-cell lymphoma line also showed relatively low efficacy of the indirect Protein A conjugates, although even here uptake varied according to antibody specificity, with greater internalization of an anti-Dr conjugate than an antidiotypic (12). The fact that

![Fig. 5. Total cell association and internalization of fluorescent liposomes by human T-cell leukemia after a 72-h incubation.](image-url)

Leukemia cells were incubated continuously for 72 h at 37°C with liposomes containing HPTS using two lipid concentrations, 2.5 µM and 25 µM. After removal of unbound liposomes, the cell-associated liposomes and liposomes at low pH were measured by fluorimetry. Results are expressed as nmol of REV (lipid), assayed by fluorescence, per 2 × 10<sup>6</sup> leukemia cells. The x-axis shows the target antibody used, where SS.1 and 14.2a are irrelevant controls, OKT11 is anti-CD2, OKT3 is anti-CD3, Leu 1 is anti-CD5, and B3/25 and OKT9 are antitransferrin receptor. •<sub>1</sub> liposomes at low pH (internalized); •<sub>r</sub>, total cell-associated liposomes.
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the differences in the current study between the direct and indirect liposomes were more marked for the growth inhibition assays than for the fluorescence assays may be due to the differences in incubation conditions of the two measurements. In order to obtain accurate fluorimetry measurements, greater concentrations of liposomes (10-fold greater than in growth inhibition) were used at shorter incubation times than in the growth inhibition treatments (4 versus 72 h). Over longer time periods, cell-associated fluorescence of SPA-REV completely disappears, as was shown by microscopy. Thus, the contents of SPA-REV are gradually lost from the cell, while the Ab-REV are retained. This may be related to early leakage of contents from the SPA-REV or to a difference in intracellular processing.

The particular choice of target antigens resulted in dramatic differences not only in amount of binding, an expected effect of antigen density, but also in the cell surface distribution of fluorescence and extent of endocytosis. The intensity and distribution of cellular fluorescence seen illustrate the requirement for significant binding, uptake, and retention of the liposomes. Thus, while anti-CD3 facilitates comparatively rapid uptake and complete internalization of cell-associated liposomes (Fig. 3B), a finding recently corroborated by Gray et al. (42), the amount of binding, endocytosis, and retention is lower than with anti-CD5 or antitransferrin receptor and insufficient for adequate drug delivery. The antitransferrin receptor-liposomes show the highest degree of cell association and endocytosis, the greatest retention of fluorescence both at 24 and 72 h, and the most potent growth inhibition. Linear correlation coefficients for the absolute amount of liposomes internalized by fluorescent assay and the IC50 for growth inhibition were 0.97 and 0.86, respectively, for SPA-REV and Ab-REV with Jurkat cells (derived from Fig. 2 and Table 1, excluding anti-CD2). It is interesting that the dual conjugate, the liposome preparation conjugated to both anti-CD5 and antitransferrin receptor, was no more effective than the antitransferrin receptor liposomes alone. This finding may be due to a limited capacity of the cells for endocytosis of the liposomes, which cannot be enhanced by binding more liposomes. It may also be due to inability to increase the number of liposomes bound beyond those already attached to the transferrin receptor. Finally, if the transferrin receptor and the CD5 antigen are internalized by similar pathways, synergy will not occur. In the latter case, increasing the potency of the encapsulated drug would be required to enhance efficacy.

The only target antigen that shows poor correlation of endocytosis by the 4-h fluorescence assay and growth inhibition assay is the CD2 (OKT11). The anti-CD2-REV show the greatest early uptake by fluorescence, but only intermediate efficacy for growth inhibition. It has been shown that endosomes formed by lymphoblasts during receptor-mediated endocytosis of various ligands may be either acidified or not, depending on the type of the ligand (43–45). It is possible that the anti-CD2-REV do not induce a high enough degree of acidification of the pinocytic or endocytic vesicle, so that the drug is not as effectively delivered to the cytoplasm. The other possibility is that over time, the contents of the anti-CD2-REV may be lost from the cytoplasm, resulting in less growth inhibition. This is supported by the results of the 72-h fluorimetry experiment (Fig. 3), which shows proportionally lower fluorescence from the cells incubated with anti-CD2-REV compared to the anti-CD5- or antitransferrin receptor-REV.

The results with the various antibody-liposome conjugates are compatible with the known characteristics of the respective antigens. The complete internalization of liposomes bound to the CD3 antigen is consistent with the known capacity of this receptor to stimulate modulation of the T3-T-cell receptor complex (46). Similarly, the CD5 antigen has also been shown to modulate when bound to antibody (47–49). This may be a disadvantage for antibody therapy alone (48), but this modulation may enhance endocytosis of immunoconjugates, such as immunotoxins (50) or liposomes, as in the current study. It is possible that the rapid cross-linking and capping of the CD5-antibody complex caused the shedding of liposomes observed with the indirect SPA-REV, while the capacity for multiligand binding of the direct Ab-REV facilitated internalization of the liposomes with modulation of the receptor, accounting for their excellent uptake. Our results differ from those of Youle and coworkers (51), who found that anti-CD3-diphtheria toxin conjugates were more effective than anti-CD5 immunotoxins with Jurkat cells. In contrast, we showed using the same cell line that the anti-CD5-REV were more effective than the anti-CD3-REV. This discrepancy may be due to differences between immunotoxins and immunoliposomes which may alter uptake, such as the multivalency of the liposomes or the larger size. The best liposome uptake was observed using the transferrin receptor, known to internalize via a coated pit pathway (13, 14) and undergo continuous cycles of endocytosis (18, 19).

These results suggest that use of a cell target known to undergo spontaneous endocytosis and recycling, like the transferrin receptor or other growth factor receptors, may markedly enhance the amount of intracytoplasmic delivery of targeted drug carriers. A similar variability in endocytic capacity of lymphocytes for antibody-targeted liposomes has previously been reported in a murine system using anti-H-2 Kβ- and anti-H-2 I-Eα antibody-targeted liposomes (52). The combination of antibody to a target antigen which is highly endocytic with a more cell-specific antibody may improve the efficacy of such targeted liposome therapy, particularly for cells like T-lymphocytes, which have been shown to have a low capacity for pinocytosis (53). Such cells have proven more resistant to antibody-targeted liposome uptake in the past (4, 5, 12). Use of a sensitive fluorescence assay like the one with HPTS will provide new information about intracellular processing of targeted liposomes and allow comparison of the fate of the aqueous contents of liposomes with that of antibody alone.

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Role of Ligand in Antibody-directed Endocytosis of Liposomes by Human T-Leukemia Cells

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