

# Antibody-directed Targeting of Liposomes to Human Cell Lines: Role of Binding and Internalization on Growth Inhibition<sup>1</sup>

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## ABSTRACT

Small unilamellar liposomes containing methotrexate or methotrexate- $\gamma$ -aspartate were conjugated to *Staphylococcus aureus* protein A and were thus able to bind cell-specific immunoglobulins for targeting to malignant human B- and T-cell lines. We were able to demonstrate enhanced protein A liposome uptake and growth inhibition by targeting with an anti-major histocompatibility complex class II antibody recognizing two different B-cell lines. The enhanced growth inhibition was specific for the targeting antibody and amounted to a 2- to 3-fold lowering of the concentration of drug required to inhibit cell growth by 50% as compared to nontargeted liposomes or liposomes targeted with an antibody not recognizing a cell surface antigen. A strong association between enhanced growth inhibition and liposome internalization as assessed by fluorescent-activated cell sorter analysis of carboxyfluorescein containing protein A liposomes was seen. By contrast, specific enhancement of growth inhibition was not seen with several anti-idiotypic antibodies or antibodies to T-cell differentiation antigens. Liposome internalization did not occur with these antibodies. Failure of growth inhibition and PA liposome internalization could not be explained by differences in cell binding of the antibody PA liposomes or the degree of protein A binding of the targeting antibody. Although the ability of the targeting antibody to bind to the cell and to protein A are important, these factors alone are not sufficient to guarantee internalization and growth inhibition. Variations in rates of internalization of various cell surface antigen-antibody complexes may account for different protein A liposome mediated cytotoxicities.

## INTRODUCTION

Liposomes, containing chemotherapeutic agents, provide the possibility of selective specific drug delivery and cytotoxicity (1). Methods of conjugating proteins (2-4) have enabled the attachment of immunoglobulins or protein A to liposomes for specific targeting. Protein A has the property of binding preferentially certain isotypes of immunoglobulins (IgG2a and IgG2b) and immunoglobulins of certain species (rabbit and murine *versus* goat which binds less well) (5). The conjugation of cell-specific antibodies to liposomes containing chemotherapeutic agents has been shown to increase cytotoxicity selectively in several murine tumor cell lines (6, 7). However, there are only a few reports in the literature examining the application of liposome technology to human cell lines (8). Specific enhanced tumor cytotoxicity could be of clinical relevance, especially in the area of tumor purging of bone marrows for autologous transplantation. In this report, we evaluate the efficacy of monoclonal antibody-targeted PA liposomes<sup>5</sup> containing methotrexate and methotrexate- $\gamma$ -aspartate in human neoplastic lymphoid cell lines.

Received 2/27/87; revised 8/10/87; accepted 8/18/87.

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<sup>1</sup> Supported by NIH Grants CA 33303, CA 33399, CA 34233, and CA 39448.

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<sup>5</sup> The abbreviations used are: PA liposome, protein A conjugated to small unilamellar vesicles; FACS, fluorescent-activated cell sorter; MHC, major histocompatibility complex; IC<sub>50</sub>, drug concentration that inhibits cell growth by 50%; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline.

## MATERIALS AND METHODS

**Liposomes.** A 10:5:1 molar mixture of phosphatidylcholine, cholesterol, and *N*-4-(*p*-maleimidophenyl)butyl phosphatidylethanolamine was suspended in a solution of 50 mM methotrexate- $\gamma$ -aspartate as previously described (9). The suspension was sonicated for 1 h in a bath sonicator (Laboratory Supplies, Hicksville, NY). Any remaining large multilamellar liposomes were removed by centrifugation in an Eppendorf 3200 centrifuge for 30 min, and unencapsulated drug was removed by gel filtration on Sephadex G-75. The vesicles were then conjugated for 18 h at 25°C with thiolated *Staphylococcus aureus* protein A in isotonic 50 mM 2(*N*-morpholino)ethanesulfonic acid-50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid-2 mM EDTA buffer, pH 6.7. The protein and lipid concentrations during conjugation were 0.25 g/liter and 2.6 mM, respectively. The conjugated vesicles were separated from unbound protein by flotation on a metrizamide gradient as described (8). Liposomes were sterilized by passing them through a 0.2- $\mu$ m polycarbonate filter, and analyzed for lipid, protein, and drug content as previously described (10). Methotrexate PA liposomes were made in a similar fashion but with a 5:5:1 ratio of the three lipids suspended in a methotrexate concentration of 25 mM.

Carboxyfluorescein PA liposomes were prepared as above but with the lipids suspended in a solution of 100 mM carboxyfluorescein instead of methotrexate- $\gamma$ -aspartate.

**Cell Lines.** Cells used included the human T-cell leukemia line CEM/VLB (obtained from Dr. W. T. Beck, Memphis, TN), the human B-cell lymphoma lines TAB (obtained from Dr. S. Smith, Stanford, CA) (11), OCI LY8 (obtained from Dr. H. Messner, Toronto, Ontario, Canada) (12), and the murine lymphoma line AKR/J SL2 (obtained from Dr. I. Bernstein, Seattle, WA).

**Antibodies.** Antibodies for targeting experiments included the anti-CD5 antibody Leu-1 (IgG2a), anti-CD7 antibodies 4H9 (IgG2a) and S91 (IgG2a) (obtained from Dr. E. Engelman, Stanford), the anti-MHC class II antibody L243 (IgG2a), and anti-B2 microglobulin antibody L368 (IgG1). The Thy-1.1 hybridoma was provided by Drs. Nowinsky and Bernstein (Fred Hutchinson Cancer Center, Seattle). The anti-idiotypic antibodies, anti-TAB (IgG1) and anti-OCI LY8 (IgG2a), were produced by fusion of the spleen cells of BALB/c mice with P3X6 8653 myeloma cells. The mice were immunized with the secreted idiotype of the hybridoma between the TAB or OCI LY8 tumor cells and K6HB5 myeloma cells (13). All antibodies were purified by double ammonium sulfate precipitations and were judged to be greater than 80% pure on sodium dodecyl sulfate polyacrylamide gel electrophoresis. The control nonbinding antibody used in the growth inhibition assays was the OCI LY8 anti-idiotypic antibody for experiments using the cell lines CEM/VLB, TAB, and AKR/J SL2, and the TAB anti-idiotypic antibody for experiments using OCI LY8.

**Growth Inhibition.** The targeting or control antibody was added at 0.1  $\mu$ g/10<sup>5</sup> cells and after 20 min at 4°C, excess antibody was removed by washing with cold RPMI 1640 twice. Aliquots of 1  $\times$  10<sup>5</sup> cells in 1 ml were added in duplicate to a 24-well plate. Increasing molar concentrations of PA liposomes or free drug in 10  $\mu$ l were added to the appropriate wells. The drug or PA liposomes were incubated with the cells continuously for the entire assay period. The cells were incubated for 72 h at 37°C, 5% CO<sub>2</sub>, and then counted on a Coulter Counter. All assays were performed in RPMI 1640 (Gibco) supplemented with 15% heat-inactivated fetal calf serum (Flow Laboratories). Results were expressed either as percentage of control cell number or as the IC<sub>50</sub>.

**Liposome Binding.** Cells (1  $\times$  10<sup>6</sup>) were incubated 20 min at 4°C with the specific antibody (100  $\mu$ l at 10  $\mu$ g/ml per 10<sup>6</sup> cells). Excess antibody was removed by washing twice with Dulbecco's PBS with 1% bovine

serum albumin. The cells were resuspended in 1 ml PBS and PA liposomes were added and incubated at 4°C for 20 min. Excess PA liposomes were removed by washing twice and then 100 µl of the nonspecific fluorescein-conjugated antibody were added as a counterstain, then washed after 20 min at 4°C. Cells were resuspended in 1 ml PBS at 4°C and analyzed on the Becton Dickinson FACS 440.

**Liposome Internalization.** PA liposomes containing carboxyfluorescein were used to demonstrate liposome internalization. Cells ( $1 \times 10^6$ /tube) were pretreated with appropriate antibodies and washed. The carboxyfluorescein PA liposomes were added at lipid concentrations equal to the predetermined  $IC_{50}$  and incubated at 4°C or 37°C for specified times, washed with cold medium, and then analyzed on the FACS.

**Antibody Binding.** The degree of antibody binding to the various cell lines was assessed by indirect immunofluorescence by using an affinity purified goat anti-mouse fluorescein-conjugated monoclonal antibody. Samples were analyzed on the FACS 440.

**Protein A Binding.** The degree of protein A binding was assessed by an ELISA in which 96-well microtiter plates (Immulon, Dynatech) were coated with protein A at a concentration of 5 µg/ml for 18 h. The plates were blocked with PBS containing 5% Carnation evaporated milk and then washed. Serial dilutions of the respective antibodies (pH 7.6) were added (1 µg/ml) and washed after a 1-h incubation. Goat anti-mouse immunoglobulin horseradish peroxidase was then added for 1 hour and washed. 2,2'-Azidobis(3-ethylbenzthiazolinesulfonic acid)-citric acid- $H_2O_2$  substrate solution was added, and color was quantitated on a Dynatech micro-ELISA reader at a wavelength of 405 nm with a reference wavelength of 630 nm.

## RESULTS

**Growth Inhibition.** We assessed the ability of various cell-specific monoclonal antibodies linked to PA liposomes containing a drug to inhibit the growth of several malignant cell lines. Two different compounds were assessed. Initially, methotrexate- $\gamma$ -aspartate was chosen for encapsulation. This pteridine antifolate is not as efficiently transported into cells as methotrexate, while it is an equally good inhibitor of dihydrofolate reductase (14). The effect from possible uptake of leaked drug is therefore insignificant (9, 15). Subsequently, methotrexate was tested as well.

Growth inhibition of B- and T-cell lines by PA liposomes with encapsulated methotrexate- $\gamma$ -aspartate bound to a variety of antibodies is shown in Table 1. The  $IC_{50}$  for free nonencapsulated methotrexate- $\gamma$ -aspartate was similar in the different human lines ( $8 \times 10^{-7}$  to  $1 \times 10^{-6}$  M), while the murine line was slightly more sensitive ( $IC_{50}$  of  $5 \times 10^{-7}$ ). The drug encapsulated in nontargeted PA liposomes was more effective in growth inhibition than free drug on all cell lines, with the greatest reduction of  $IC_{50}$  (7.5-fold) seen in the murine line. With the targeted PA liposomes, in the murine example as shown previously (9), there was a marked further reduction in the  $IC_{50}$  of 12.5-fold compared to nonspecific antibody-targeted PA liposomes. In the human lines, only a modest targeting effect was seen using the anti-MHC class II monoclonal antibody, L243 in one B-cell lymphoma line with an approximate 2-fold reduction in the  $IC_{50}$  compared to PA liposomes targeted with a nonspecific antibody. This reduction in  $IC_{50}$  was seen reproducibly in three separate experiments. No reduction in  $IC_{50}$  was seen when targeting was performed with an anti-idiotypic antibody against this B-cell line or with antibodies against CD5 or CD7 in the T-cell line.

Because of the minimal growth inhibition seen in the human cell lines with most of the monoclonal antibodies tested, methotrexate, a chemotherapeutic agent more potent in free form than the  $\gamma$ -substituted derivative, was encapsulated into the PA

Table 1 Growth inhibition of leukemia cell lines by protein A liposomes

Cells ( $1 \times 10^5$ ) were washed, reacted with designated antibodies (100 µl of 1 µg/ml), washed again, and exposed to varying molar concentrations of liposomes or free drug. After 72 h at 37°C, the cell numbers were counted on a Coulter Counter. Exposures to drug and PA liposomes were continual. No enhanced targeting effect was seen at shorter drug or liposome exposure times (data not shown).

Cell line	Antibody	$IC_{50}$ (M) <sup>a</sup>	
		Free drug	PA liposome
Methotrexate- $\gamma$ -aspartate CEM/VLB (human T)	Control <sup>b</sup>	$8 \times 10^{-7}$	$2.5 \times 10^{-7}$
	Anti-CD7 (4H9)		$2.5 \times 10^{-7}$
	Anti-CD7 (S91)		$2.5 \times 10^{-7}$
	Anti-CD5 (Leu-1)		$2.0 \times 10^{-7}$
TAB (human B)	Control	$1 \times 10^{-6}$	$8 \times 10^{-7}$
	Anti-idiotypic		$9 \times 10^{-7}$
	Anti-MHC class II (L243)		$5 \times 10^{-7}$
AKR/J SL2 (murine T)	Control	$5 \times 10^{-7}$	$7.5 \times 10^{-8}$
	Thy-1.1		$5 \times 10^{-9}$
Methotrexate CEM/VLB (human T)	Control	$8 \times 10^{-9}$	$5 \times 10^{-7}$
	Anti-CD5 (Leu-1)		$5 \times 10^{-7}$
	Anti-B2 microglobulin		$4 \times 10^{-7}$
TAB (human B)	Control	$2 \times 10^{-8}$	$6 \times 10^{-7}$
	Anti-idiotypic		$6 \times 10^{-7}$
	Anti-MHC class II (L243)		$3 \times 10^{-7}$
OCI LY8 (human B)	Control	$4 \times 10^{-8}$	$9 \times 10^{-7}$
	Anti-idiotypic		$9 \times 10^{-7}$
	Anti-MHC class II (L243)		$3 \times 10^{-7}$

<sup>a</sup> Mean standard deviation between duplicates in all experiments was less than 5%.

<sup>b</sup> Murine class matched nonbinding antibody.

liposomes. Table 1 also shows the  $IC_{50}$  for human cell lines when methotrexate instead of methotrexate- $\gamma$ -aspartate was incorporated in the PA liposomes. The cell lines were clearly more sensitive to free methotrexate than to methotrexate- $\gamma$ -aspartate. A targeting effect was again seen with the anti-MHC class II antibody. In this case there was a 3-fold reduction in  $IC_{50}$  for OCI LY8 and a 2-fold reduction for TAB. There was an increase in  $IC_{50}$  for encapsulated methotrexate as compared to free methotrexate which was not seen for encapsulated methotrexate- $\gamma$ -aspartate as compared to free methotrexate- $\gamma$ -aspartate. In contrast to methotrexate- $\gamma$ -aspartate, free methotrexate is more effective than drug encapsulated into liposomes because of its higher rate of influx. However, the  $IC_{50}$  of encapsulated methotrexate is similar to that of encapsulated methotrexate- $\gamma$ -aspartate, since cell entry of encapsulated drug depends on the uptake of the liposomes. The antibodies used for targeting did not alter the cytotoxicity induced by either free methotrexate or free methotrexate- $\gamma$ -aspartate (data not shown).

Thus, specific growth inhibition in the human cell lines by PA liposomes containing methotrexate or methotrexate- $\gamma$ -aspartate was seen only when an anti-MHC class II antibody was used for targeting the liposomes. Fig. 1 shows the PA liposome dose-response effect when the anti-MHC class II antibody was used for targeting. An antibody dose-response effect was also seen with this targeting antibody (data not shown). Note that the  $IC_{50}$  for free methotrexate was lower than the  $IC_{50}$  for anti-MHC class II antibody-targeted PA liposomes containing methotrexate. In this case enhancement of targeting refers to a reduction in  $IC_{50}$  for specifically targeted as compared to non-

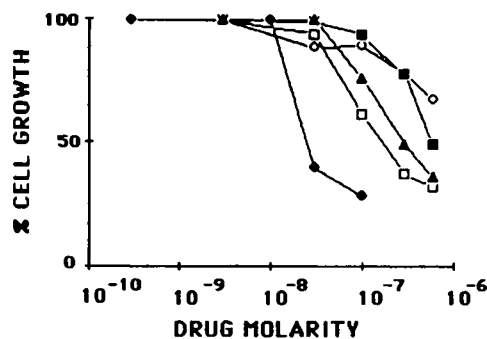


Fig. 1. Specific enhanced growth inhibition effect by class II antibody-targeted PA liposomes. Growth inhibition was assessed for the OCI LY8 cell line (see "Materials and Methods") and expressed as percentage of control cell growth. Specific enhanced growth inhibition was seen with both methotrexate and methotrexate- $\gamma$ -aspartate containing PA liposomes (although results shown are with methotrexate encapsulated PA liposomes). Shown is a representative experiment with free methotrexate ( $\blacklozenge$ ), nonspecific antibody-PA liposomes ( $\diamond$ ), anti-OCI LY 8 idio-type-PA liposomes ( $\blacksquare$ ), anti-MHC class II-PA liposomes ( $\square$ ), anti-idio-type and anti-MHC class II-PA liposomes ( $\blacktriangle$ ).

specifically targeted drug containing PA liposomes. For the other antibodies used on the human cell lines, the curves in Fig. 2 were representative of the absence of targeting effect seen over a 1000-fold increment in the concentration of the targeting antibody. Because of this result, several variables were examined to explain why only the anti-MHC class II antibody was effective in producing enhanced growth inhibition.

**Comparison of Antibody Binding.** A comparison of the fluorescent intensity of monoclonal antibody binding to the various cell lines was made in order to correlate antibody-directed PA liposome-mediated growth inhibition and antibody-cell binding. Table 2 shows the mean fluorescent intensity (using the same fluorescein-labeled second step antibody) for the various antibodies used for targeting on the different cell lines. The anti-MHC class II antibody bound well to both B-cell lines, although there were other antibodies that exhibited higher degrees of binding to these cell lines. No correlation between specific PA liposome growth inhibition effect and mean fluorescence was seen.

**Comparison of Protein A Binding.** To detect any correlation between specific monoclonal antibody directed PA liposome mediated growth inhibition and the ability of the monoclonal antibody to bind protein A, an ELISA binding assay was performed (Table 2). The antibody Thy-1.1 used on the AKR/J SL2 cell line eliciting a marked enhanced specific growth-inhibiting effect binds to protein A most avidly. There were several other antibodies which bound protein A well, yet only the anti-MHC class II antibody produced enhanced growth inhibition compared to nonspecifically targeted PA liposomes. Antibodies which did not bind to protein A, such as anti-B2 microglobulin antibody, regardless of brightness to which they bound to the target were not able to elicit specific enhanced PA liposome growth inhibition.

**Demonstration of PA Liposome Binding.** To demonstrate that the PA liposomes were binding to the cell via the targeting antibodies, an indirect assay was used, based on the binding of a fluoresceinated nonspecific antibody to "unoccupied" protein A sites on PA liposomes bound to the cell surface, Fig. 3 shows a shift in the mean fluorescence in both a murine and human cell line from the addition of a nonspecific fluorescein-conjugated IgG2a antibody to the cell-specific antibody-PA liposome complex. This shift did not occur when this antibody was added to either a cell-antibody (no PA liposome) complex or to a cell-PA liposome (no specific antibody) complex. A shift in mean fluorescence indicating PA liposome binding to the cell via the

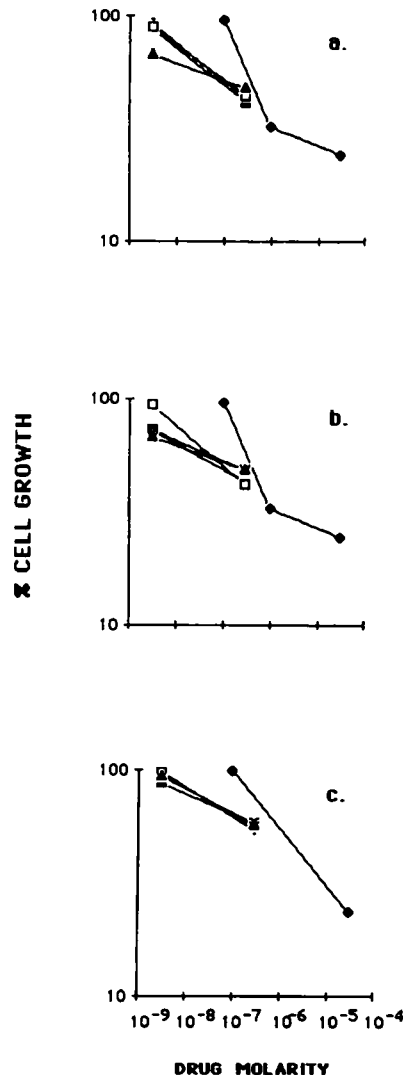


Fig. 2. Effect of targeting antibody concentration on growth inhibition. Cells ( $1 \times 10^5$ ) were washed and incubated with the varying antibody amounts at 4°C for 20 min, washed, and then exposed to varying molar concentrations of PA liposomes or free drug (methotrexate- $\gamma$ -aspartate). After a 72-h incubation at 37°C, samples were counted on a Coulter Counter. Exposure to drug and PA liposomes was continual. Data are shown for the CEM cell line but are similar to data from other lines (see in Table 1) with antibodies that were unable to produce specific enhanced growth inhibition. In all curves, free methotrexate- $\gamma$ -aspartate ( $\blacklozenge$ ), nonspecific antibody, 1  $\mu$ g, PA liposome ( $\blacktriangle$ ). In a, b, and c, PA liposomes are targeted with S91, 4H9, and Leu-1, respectively, at 1  $\mu$ g ( $\diamond$ ), 0.1  $\mu$ g ( $\blacksquare$ ), and 0.01  $\mu$ g ( $\square$ ) (per  $10^5$  cells).

specific antibody occurred on the TAB cell line for PA liposomes targeted by both the anti-idio-type and anti-MHC class II antibodies. However, from the growth inhibition experiments discussed above, only the anti-MHC class II antibody resulted in specific growth inhibition. Thus, cell immunoglobulin-PA liposome binding can occur without enhancing growth inhibition.

The histograms (shown on a logarithmic scale) in Fig. 3 indicate that the degree of cell binding for PA liposomes targeted by the anti-idio-type antibody and the anti-MHC class II antibody was similar in magnitude to the amount of binding seen with the anti-Thy-1.1 antibody on the murine cell line. Thus, degree of PA liposome binding to the cell does not account for the marked differences in ability of the anti-Thy-1.1 antibody as compared to the other two antibodies to produce PA liposome mediated growth inhibition.

**Demonstration of PA Liposome Internalization.** In order to explain the discrepancy between binding and growth inhibition,

Table 2 Comparison of degree of antibody binding to cells and antibody binding to protein A

Cell line	Antibody	Antibody-cell binding (fluorescence) <sup>a</sup>	Antibody-protein A binding (A <sub>405</sub> ) <sup>b</sup>
CEM/VLB	Anti-B2M	133	0.08
	Anti-CD5 (Leu-1)	70	0.363
	Anti-CD7 (4H9)	105	0.435
	Anti-CD7 (S91)	105	0.430
TAB	Anti-MHC class II	108	0.273
	Anti-TAB idiotype	64	0.15
OCI LY8	Anti-MHC class II	102	0.273
	Anti-OCI LY8 idiotype	145	0.246
AKR/J SL2	Anti-Thy-1.1		0.633

<sup>a</sup> Mean fluorescent intensity was assessed by FACS after indirect staining with a goat anti-mouse fluorescein conjugate. Binding is shown as mean fluorescent units minus control nonbinding antibody.

<sup>b</sup> Protein A binding was assessed by an ELISA assay, as per "Materials and Methods." Background absorbance, as detected on the detector antibody (low protein A binder) was subtracted from sample absorbance. Absorbance values shown represent a 1:16 dilution for all antibodies.

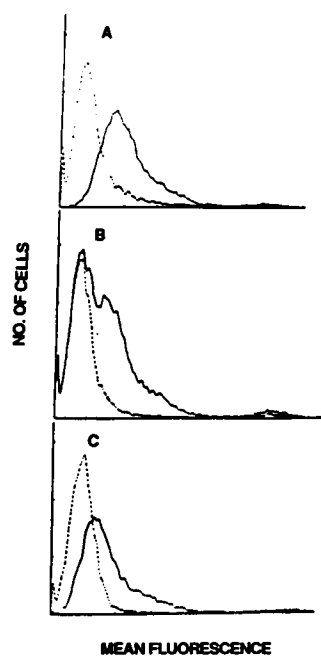


Fig. 3. Demonstration of liposome binding. A nonspecific fluoresceinated anti-idiotype antibody (IgG2a) was utilized to indicate PA liposome binding to cell in a successive layering assay. Specific or control antibody ( $1 \mu\text{g}/10^6$  cells) was added to the cells at  $4^\circ\text{C}$ . PA liposomes were then added. After washing, indicator fluoresceinated nonspecific antibody was added and excess was washed off. —, log scale fluorescence seen when all components of the layered complex were added; ···, decreased fluorescence seen when one of the components (specific targeting antibody or PA liposome) was excluded. PA liposomes targeted with (A) the Thy-1.1 antibody on the AKR/J SL2 cell line; (B) the anti-TAB idiotype antibody on the TAB cell line; (C) the anti-MHC class II antibody on the TAB cell line.

we determined whether the PA liposomes associated with cells were internalized. To do this we made use of the self-quenching effect of carboxyfluorescein when it is encapsulated at high concentrations within the internal aqueous spaces of liposomes (16, 17). The release of carboxyfluorescein into the cell, after successful uptake, results in measurable increased fluorescence, due to dilution and dequenching. The background immunofluorescence for cells binding carboxyfluorescein PA liposomes targeted with the antibodies at  $4^\circ\text{C}$  is shown in Fig. 4. Since the process of internalization is inhibited at  $4^\circ\text{C}$ , this fluorescence did not change significantly when assessed at 90 or 150 min (data not shown). At  $37^\circ\text{C}$ , there was an increase in mean fluorescence for the cells targeted with the anti-MHC class II

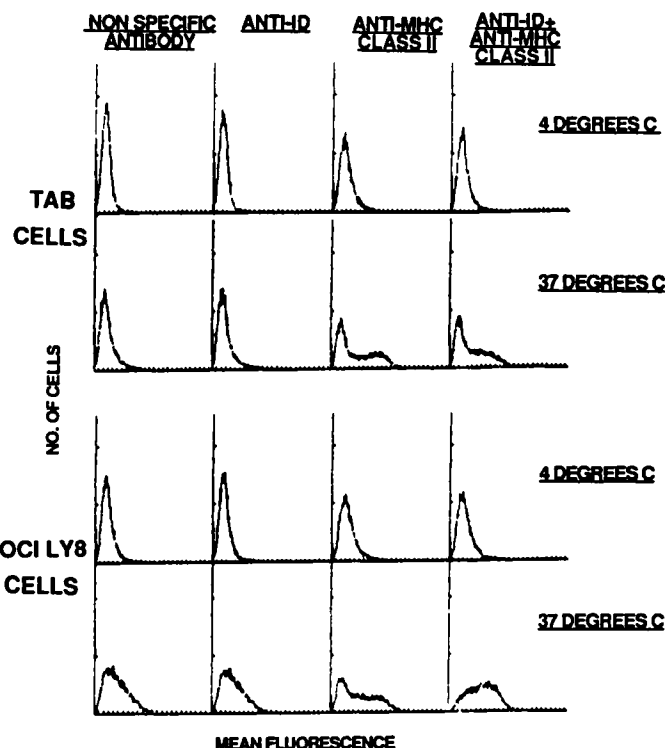


Fig. 4. Demonstration of liposome uptake. PA liposomes containing carboxyfluorescein were utilized to assess liposome internalization of two B-cell lines. The self-quenching effect of carboxyfluorescein at high concentration in the PA liposome is overcome when carboxyfluorescein is released into the cell. Cells ( $1 \times 10^6$ ) were reacted with antibody ( $100 \mu\text{l}$  of  $10 \mu\text{g}/\text{ml}$ ) for 20 min at  $4^\circ\text{C}$ , washed, and then the PA liposomes were incubated for 20 min at  $4^\circ\text{C}$  and washed. One set of samples was kept at  $4^\circ\text{C}$  (to minimize receptor-mediated endocytosis) and one set was incubated at  $37^\circ\text{C}$ , for 30 min. Samples were analyzed on the FACS 440. ANTI-ID, anti-idiotype.

antibody for both the OCI LY8 and the TAB cell lines. This indicates internalization of the liposome with subsequent release of carboxyfluorescein into the cytoplasm. This increase in fluorescence was not seen when targeting was performed with a nonspecific antibody or with anti-idiotype antibodies against either cell line. Thus, a correlation was seen between monoclonal antibody-directed PA liposome internalization and the ability of the antibody to produce specific PA liposome-mediated growth inhibition. It is noted that the greatest PA liposome internalization was seen in the OCI LY8 line when both the anti-MHC class II and anti-idiotype antibodies were used together for targeting. The further increase seen in liposome internalization for the two antibodies together did not result in further enhancement of growth inhibition compared to anti-MHC class II targeting alone (Fig. 1).

## DISCUSSION

The data in this paper show that drug-containing small unilamellar liposomes which are conjugated to protein A can be targeted with a monoclonal antibody to result in specific growth inhibition of human B-cell lines (Table 1). Although examples are present in the literature that show enhanced cytotoxicity with targeted PA liposomes in murine cell lines (16, 18), to date specific liposome cytotoxicity in human lymphoma cell lines has been unsuccessful (8).

We have demonstrated that the enhanced growth inhibition seen by targeting with the anti-MHC class II monoclonal antibody correlates with an increase in PA liposome internalization (Fig. 4). This enhanced growth inhibition and PA liposome internalization does not occur with antibodies directed against

other cellular surface antigens such as the immunoglobulin idiotype. The degree of growth inhibition and PA liposome internalization seen with anti-idiotype antibodies and antibodies to T-cell differentiation antigens was equivalent to that seen with targeting by control nonbinding antibodies. This growth inhibition and internalization was probably related to nonspecific endocytosis of the PA liposomes on their own and not through an antibody-mediated pathway. This has been previously demonstrated for AKR/J SL2 cells with both direct antibody-liposome conjugates and PA liposomes (8, 9). Similar growth inhibition was seen when cells were incubated with PA liposomes without a targeting antibody (data not shown).

When the anti-MHC class II antibody was used for targeting, a dose-response effect was seen for both the amount of PA liposomes and the amount of targeting antibody used. In contrast, with all other antibodies used for targeting, no dose response could be seen over a 1000-fold range in targeting antibody concentration. Increasing amounts of antibody were thus unable to increase the intrinsically minimal antibody-mediated PA liposome internalization and growth inhibition by these antibodies. A concentration effect for antibody was thus seen with the antibody that was internalized well and not with those that were not internalized well. This is similar to results reported in a previous publication that used a murine model (8).

Although the enhancement of growth inhibition seen with the specific as compared to the nonspecific monoclonal antibody-targeted PA liposomes (approximately 3-fold) was not as dramatic as that seen with some monoclonal antibody-toxin conjugates (19, 20), a reproducible and specific effect was seen.

We have investigated some of the factors regulating the ability of monoclonal antibody targeted PA liposomes to produce a cell specific growth inhibitory effect. In order to understand why the specific growth inhibitory effect was seen with certain monoclonal antibodies (anti-MHC class II) and not with others (anti-idiotypes and antibodies to T-cell differentiation antigens), we assessed the degree of cell binding and protein A binding of the targeting antibody. Although the binding of the anti-MHC class II monoclonal antibody was high on both cell lines where a specific growth-inhibitory effect was seen (Table 2), the binding of some of the other monoclonal antibodies was similarly high. Similarly, protein A binding could not distinguish between antibodies which were able to mediate targeting and those which were not (Table 2). Clearly, for an antibody to enhance PA liposome-mediated growth inhibition, both cell surface binding and protein A binding must be above a critical level. However, these two factors alone did not assure that enhanced growth inhibition would result.

Despite the ability of PA liposomes to bind to cells via an anti-idiotype targeting antibody (Fig. 3), enhanced growth inhibition did not occur as it did with the anti-MHC class II antibody. The histograms in Fig. 3 show that the degree of PA liposome binding to the TAB cell line was comparable for targeting with either the anti-idiotype or anti-MHC class II antibodies. Furthermore, the degree of PA liposome binding through either of these antibodies was approximately as great as with the antibody which produces the most marked example of specific enhanced growth inhibition (anti-Thy-1.1 with the AKR/J SL2 murine cell line). However, increased PA liposome internalization did not occur with the anti-idiotype antibody as it did with the class II antibody (Fig. 4). The defect was clearly in PA liposome internalization for the anti-idiotype antibody PA liposome complex.

Antibody isotype is not a probable explanation for the differ-

ences in antibody-mediated liposome internalization. Although the TAB anti-idiotype was an IgG1 and the anti-MHC class II was an IgG2a, a defect in internalization was also seen with the OCI LY8 cell line and its IgG2a anti-idiotype (Fig. 4).

No additional effect on growth inhibition was seen when targeting was performed with both an antibody directed against a class II MHC determinant (where internalization was demonstrated) and an anti-idiotype antibody (where internalization on its own did not occur). The internalization data (Fig. 4) suggested that for the OCI LY8 cell line, more PA liposome internalization occurred when both the anti-idiotype antibody and the anti-MHC class II antibody were used together for targeting. The explanation for this discrepancy is unclear; however, the increase in internalization seen with the combination of antibodies may not be enough to cause augmented growth inhibition.

It is significant that only the antibody to the class II MHC determinant resulted in enhanced PA liposome internalization and growth inhibition in both B-cell lines tested. Other investigators have shown that PA liposomes targeted to class II determinants on murine B-cell lines result in increased liposome uptake and cellular cytotoxicity (7, 21). This may suggest a special role for class II determinants on B-cells for MHC-associated antigen endocytosis and processing.

An analogy can be drawn between specific growth inhibition with antibody-directed PA liposomes and antibody-toxin conjugates. Specific tumor cell lysis has been described with several different antibody-toxin conjugates, with the most marked examples being with immunotoxins directed against the immunoglobulin idiotype or isotype of a murine B-cell tumor (22, 23) or against the Thy-1 antigen on T-cells and T-cell leukemias (24-26). As with liposomes, variability in target cell sensitivity to immunotoxins has been documented (24, 27). Factors responsible for this variability have been described and include density of cell surface antigen (28), the rate of endocytosis of the antigen-immunotoxin complex (29), and the isotype of the targeting antibody (29, 30). Although we have not found the degree of targeting antibody binding (as measured by mean fluorescent intensity) and antibody isotype to be significant factors, our results concur with the importance of antigen-antibody endocytosis as a major parameter influencing effective liposome growth inhibition.

The specific growth inhibition seen by using anti-idiotype antibodies to target PA liposomes was not as marked as was seen by several authors when anti-idiotypes were used to target immunotoxins (21, 23, 31). Some immunotoxins are exquisitely effective in inhibiting cellular protein synthesis and only one ricin A chain can completely inhibit protein synthesis and kill the cell (32). Although we have shown that anti-idiotype antibodies were not internalized as rapidly as the anti-MHC class II antibody, some internalization was seen (Fig. 4). Probably, the difference in growth inhibition seen with anti-idiotype-targeted PA liposomes and anti-idiotype-targeted immunotoxins is stoichiometric with sufficient internalization occurring to deliver lethal amounts of immunotoxin but insufficient amounts of chemotherapeutic drug to kill the cell.

We have demonstrated that PA liposomes can be targeted with an anti-MHC class II monoclonal antibody in human B-cells to result in specific PA liposome internalization and cellular growth inhibition. A correlation between antibody-directed PA liposome internalization and enhanced growth inhibition was obtained. Cell surface and protein A binding of the targeting antibody are necessary but not sufficient factors for determining specific growth inhibition. The ability of the

targeted antigen to perform receptor-mediated endocytosis seems to be important and may limit the use of PA liposomes for cytotoxic purposes to certain targeting antibodies. Further clarification of these factors are necessary in order to optimize the utility of liposomes for specific cellular cytotoxicity.

#### ACKNOWLEDGMENTS

We are grateful to Dr. L. Leserman for his helpful advice and assistance and to Emily Chatfield, Phyllis Bussey, and Carolyn Wells for preparation of the manuscript.

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