Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 246–250, January 1987
Medical Sciences

Selective killing of T lymphocytes by phototoxic liposomes

(drug delivery/photochemotherapy)

SHRISHAILAM YEMUL*, CAROLE BERGER†‡, ALISON ESTABROOK§, SYLVIA SUAREZ†, RICHARD EDELSON†, AND HAGAN BAYLEY*¶

Departments of *Biochemistry, †Dermatology, ‡Pathology, and §Surgery, College of Physicians and Surgeons, Columbia University, New York, NY 10032

Communicated by Aaron B. Lerner, September 8, 1986

ABSTRACT **Two-fold specificity in drug delivery obtained through (*i*) the localized activation of drugs by physical means and (*ii*) the attachment of drugs to proteins that bind to target cells might be used for highly selective cancer chemotherapy or for immunosuppression. Toward this end, a monoclonal antibody against an antigen on the surface of T lymphocytes was covalently attached to liposomes containing a phototoxic drug, pyrene, bound to the lipid bilayer. When unfractionated peripheral blood lymphocytes, or B- and T-cell lines, were irradiated after treatment with these liposomes, T cells were killed while B cells were spared, demonstrating the validity of the approach in a simple *in vitro* assay.**

The ability to limit the site of action of drugs to specific cells has been a goal long sought by pharmacologists. In particular, attempts have been made to develop highly selective cytotoxic agents for use in cancer chemotherapy or as immunosuppressants. Recent progress in this area has relied upon the use of proteins, such as hormones, lectins, and antibodies that bind to receptors on the surfaces of the selected cells, to guide drugs, including plant and animal toxins, to their targets (1–6). Such protein–drug conjugates have been used with success to kill selectively cells in culture and in bone marrow for transplantation. For example, daunomycin conjugated directly to antibody molecules destroyed murine lymphoid tumors *in vitro* (7). Antibody-bearing liposomes containing antimetabolites (8, 9) and antibody–toxin conjugates that are effective *in vitro* also have been prepared (5).

Antibody–drug conjugates also have been used in attempts to eradicate tumors in experimental animals. For example, methotrexate conjugated to tumor-specific antibodies prolonged the survival of mice that had received melanoma or lymphoma cells (10). Actinomycin D carried by antibody-bearing liposomes was partly effective against peritoneal mammary tumor cells in mice and was of more limited efficacy against subcutaneous tumors (11). The plant toxin ricin attached to antibodies produced prolonged remission of B-cell leukemia in mice (12). The success of antibody–drug conjugates *in vivo* has been limited by their often poor ability to penetrate solid tumors and by variable expression of tumor antigens. Nevertheless, considering the general lack of recent progress in cancer therapy, drug delivery remains an attractive proposition (1–6).

Our goal has been to still further increase the selectivity of drugs by combining a second degree of specificity, physical activation, with that obtained with a protein-targeting agent. Heat (13, 14), light (15–19), or magnetism (20, 21) have been used to activate drugs in particular tissues, and magnetism has been used to concentrate in tumors drugs held in microspheres (22). Only a subset of the cells in the region subjected to physical activation would be destroyed if the drug were delivered to them as a protein conjugate. Light-activated drugs are especially attractive; highly toxic short-lived species can be generated photochemically at specific times in well-defined doses and locations (23, 24).

Previous exploration of two-fold specificity in drug delivery has been limited. Mosley and colleagues (15) showed that pyrene incorporated into low density lipoprotein is selectively phototoxic towards cells bearing the lipoprotein receptor. Here we have generalized the approach by making use of antibodies, and we demonstrate its validity *in vitro*, using the T cells in human peripheral blood lymphocytes (PBL) and a T-lymphoblast cell line as our targets. While this work was in progress, two groups have also been successful in this area, both using hematoporphyrins directly linked to antibodies (17–19).

MATERIALS AND METHODS

Antibodies. BE3, a homolog of OKT1 and T101 (25, 26), is a mouse monoclonal antibody (γ1 heavy chain) that recognizes an epitope expressed on at least 90% of the T lymphocytes in human peripheral blood (27). The antigen recognized by BE3 is also expressed on a variable percentage of abnormal B cells in some patients with B-cell chronic lymphocytic leukemia but is absent from normal B lymphocytes. BE3 was purified from ascites fluid by gel filtration using Sephacryl S-200, followed by ion-exchange chromatography on DEAE cellulose. Mouse immunoglobulin G fractions (mIgG) were obtained from Sigma (I 5381). Both BE3 and mIgG were shown to be free of contaminating serum proteins by NaDodSO$_4$/PAGE.

Cells. PBL were isolated from normal donors by flotation on Ficoll/Hypaque (28). MOLT-4, a T-lymphoblast cell line, and the Epstein–Barr virus-transformed B-cell line GM 1056A were obtained from the National Institute of General Medical Science Human Genetic Cell Repository.

Preparation of Liposomes with Covalently Attached Antibodies. BE3 was trace-labeled with ^{125}I by the chloramine-T method and mixed with unlabeled BE3 at a ratio of 1:100 (mol/mol). The antibody molecules were then derivatized with *N*-succinimidyl-3-(2-pyridyldithio)propionate (29). Approximately four pyridyldithiopropionyl groups were introduced per IgG molecule as assayed by 2-pyridinethiol release upon reduction with dithiothreitol (30). This extent of mod-

Abbreviations: PBL, unfractionated human peripheral blood lymphocytes; PHA, phytohemagglutinin; mIgG, irrelevant mouse IgG; PtdEtn, phosphatidylethanolamine; PtdCho, phosphatidylcholine; NBD-Pam$_2$-PtdEtn, *N*-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]dipalmitoyl L-α-PtdEtn; PyPam-PtdCho, 3-palmitoyl-2-[1-pyrenedecanoyl] L-α-PtdCho; PyPam$_2$-PtdEtn, *N*-[1-pyrenesulfonyl]-Pam$_2$-PtdEtn; BE3-NBDL or mIgG-NBDL, liposomes containing the lipid NBD-Pam$_2$-PtdEtn with antibody BE3 or mIgG bound to their surfaces; BE3-PyL or mIgG-PyL, liposomes containing the lipid PyPam-PtdCho, with antibody BE3 or mIgG bound to their surfaces.

¶To whom reprint requests should be addressed at: Howard Hughes Medical Institute, Columbia University, 722 West 168th St., New York, NY 10032.

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ification was optimal for subsequent attachment to liposomes and did not affect BE3 binding to lymphocytes. After removal of the pyridinethiol group by treatment with 25 mM dithiothreitol at pH 5.0 and 25°C for 10 min and immediate gel filtration in deoxygenated buffer, the protein (0.16 mg/ml) was coupled at once to the liposomes (see below) essentially as described by Martin and Papahadjopoulos (31). The protein/lipid ratio was 1:30 (wt/wt). When attempts were made to couple more protein to the liposomes, they tended to aggregate and flocculate.

Liposomes were prepared on the day of use by reversed-phase evaporation (32) using 47.5% (mol/mol) cholesterol, 45% (mol/mol) phosphatidylcholine (PtdCho) from egg yolk, 5% (mol/mol) *N*-[4-(*p*-maleimidophenyl)butyryl] phosphatidylethanolamine synthesized as described (ref. 31; it is the head group of this lipid that reacts covalently with the thiolated antibody), and 2.5% (mol/mol) 3-palmitoyl-2-[1-pyrenedecanoyl] L-α-phosphatidylcholine (PyPam-PtdCho; KSV, Helsinki, Finland). Before coupling, the suspension was extruded successively through 0.4-μm and 0.2-μm polycarbonate filters.

After coupling, the proteoliposomes were separated from unattached protein by passage through Sepharose CL-4B at 4°C. Before use, the column (40 × 1 cm) was loaded with 4 ml of sonicated soybean lipids (asolectin) at 10 mg/ml, eluted, and then equilibrated in phosphate-buffered saline (10 mM sodium phosphate/150 mM NaCl, pH 7.4). Usually ≈70% of the antibody was eluted with the liposomes. To remove uncoupled protein aggregates, the proteoliposomes were finally subjected to Ficoll flotation (see below).

The antibody–liposome conjugates were stored for up to 12 months at 4°C either after the addition of 0.025% NaN_3 or after sterilization by filtration through a 0.45-μm polysulfone membrane (Acrodisc, Gelman). Before use they were dialyzed (if they had been stored with NaN_3), subjected once again to Ficoll flotation, filtered (as above), and diluted to ≈10 μg of IgG per ml (10 μM pyrene) with PBS.

Liposomes containing the phototoxic lipid PyPam-PtdCho with mIgG bound to their surfaces (mIgG-PyL) were prepared in parallel by an analogous procedure. BE3-NBDL and mIgG-NBDL, liposomes containing the fluorescent lipid *N*-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]dipalmitoyl L-α-phosphatidylethanolamine (NBD-Pam_2-PtdEtn) (Molecular Probes, Plano, TX), were also prepared similarly. PyPam-PtdCho was replaced with the same mole fraction of NBD-Pam_2-PtdEtn. In one experiment (see Table 1), liposomes containing the lipid *N*-[1-pyrenesulfonyl]dipalmitoyl L-α-phosphatidylethanolamine ($PyPam_2$-PtdEtn) (Molecular Probes) were used. These contained the following: 45% (mol/mol) egg-yolk PtdCho, 45% (mol/mol) cholesterol, 5% (mol/mol) *N*-[4-(*p*-maleimidophenyl)butyryl] PtdEtn, and 5% (mol/mol) $PyPam_2$-PtdEtn. The liposomes containing $PyPam_2$-PtdEtn were extruded through 0.4-μm, 0.2-μm, and 0.1-μm polycarbonate filters before coupling with antibodies.

Ficoll Flotation of Proteoliposomes. A 1-ml portion of the liposome suspension was mixed with 1 ml of Ficoll solution [25% (wt/vol) in PBS] and overlaid in a centrifuge tube with 7 ml of 10% Ficoll, 1 ml of 5% Ficoll, and finally 1 ml of PBS. After centrifugation at 10,000 × *g* for 40 min at 4°C, the liposomes were collected from the interface between the 5% Ficoll and the PBS (33). Approximately 60% of the protein appeared in this fraction during the initial purification.

Irradiation of Cells After Treatment with Liposomes. PBL were washed with PBS and resuspended at 10^7 cells per ml with liposomes in the same buffer giving final concentrations of ≈10 μg of IgG/ml and 10 μM pyrene [measured by fluorescence at 377 nm (excitation 340 nm) after solubilization in 2% (wt/vol) $NaDodSO_4$]. The ratio of IgG to pyrene varied slightly between the liposomes containing PyPam-PtdCho with antibody BE3 bound to their surface (BE3-PyL) and the mIgG-PyL. In the experiments reported here, the samples treated with BE3-PyL contained slightly less and never more antibody than did the control samples containing mIgG-PyL. After 1.5 hr at 4°C, the cells were washed twice by centrifugation and resuspension in PBS at 4×10^6 cells per ml. They were then irradiated at 320–400 nm for 40 min at 23°C in the wells of microtiter dishes (2×10^5 cells in 50 μl per well). The total light dose was 8 J/cm^2.

Thymidine Incorporation After Stimulation with Phytohemagglutinin (PHA). After irradiation, the cells were supplemented with RPMI 1640 medium (150 μl per well) containing 20% fetal calf serum and 2% PHA (omitted in unstimulated controls). After three days in culture, [^{3}H]thymidine was added (1 μCi to each well; 1 Ci = 37 GBq), and 6 hr later the incorporation of ^{3}H into DNA was measured.

Treatment of Cell Lines. The cell lines GM 1056A and MOLT-4 were treated with BE3-PyL under the same conditions as the PBL, except that the liposomes contained $PyPam_2$-PtdEtn rather than PyPam-PtdCho and they were 100 nm in diameter. After irradiation, spontaneous [^{3}H]thymidine uptake was measured over 24 hr.

Blocking the BE3 Antigen. PBL (10^7 cells per ml) in PBS were treated with BE3 (100 μg/ml) or control ascites fluid containing irrelevant mIgG (γ1) (100 μg of protein per ml) for 1.5 hr at 4°C. The cells were centrifuged and resuspended at the same density in PBS containing BE3-PyL (10 μg/ml) in the presence of BE3 or mIgG ascites (100 μg/ml). After a further 1.5 hr at 4°C, the cells were washed and irradiated as described above.

Removal of the BE3 Antigen by Modulation. PBL (10^7 cells per ml) were treated with BE3 (100 μg/ml) or control ascites fluid containing irrelevant mIgG (γ1) (100 μg of protein per ml) for 17 hr at 37°C. Initially, 59% of the cells were BE3-positive as determined by cytofluorography. After 17 hr, <1% of the cells treated with BE3 retained BE3 on their surfaces or bound fresh BE3, while 55% of the cells treated with mIgG remained BE3-positive. The cells were then treated with BE3-PyL as described above in the presence of fresh BE3 or mIgG ascites (100 μg/ml).

RESULTS

Preparation of Phototoxic Liposomes. Large unilamellar lipid vesicles (liposomes) containing the phototoxic molecule pyrene were constructed with a mouse monoclonal antibody, BE3 (ref. 27), covalently bound to their surfaces. BE3 binds to an antigen found on ≈90% of normal T lymphocytes. Pyrene was among our early choices of photoactivatable agents, as Mosley and colleagues (15) had found it to be phototoxic towards fibroblasts when incorporated into low density lipoprotein. Accordingly, the PtdCho analog PyPam-PtdCho, in which the 2-acyl chain is 1-pyrenedecanoate, was incorporated into the bilayer of the liposomes. The vesicles were nominally 200 nm in diameter, and each contained ≈10,000 pyrenyl groups and carried 20–25 antibody molecules.

Phototoxic Liposomes Conjugated to the Antibody BE3 Bind Selectively to T Lymphocytes. The liposomes were first tested to determine whether they would bind selectively to T cells in PBL. Lymphocytes were incubated with the liposomes and then treated with goat anti-mouse F(ab')$_2$ fragments conjugated to fluorescein, before assay by cytofluorography (Fig. 1 *Left*). Seventy percent (±5%) of the cells bound BE3-PyL. The same fraction bound the T cell-specific antibodies OKT1 and OKT3. For example, when BE3 and OKT3 were compared, 67 ± 7% of the cells bound BE3 and 72 ± 4% bound OKT3 (n = 6). In a second assay PyPam-PtdCho was replaced with NBD-Pam_2-PtdEtn, a fluorescent lipid analog (34), and binding was measured directly by cytofluorography with a similar result (Fig. 1 *Right*). Liposomes to which IgG

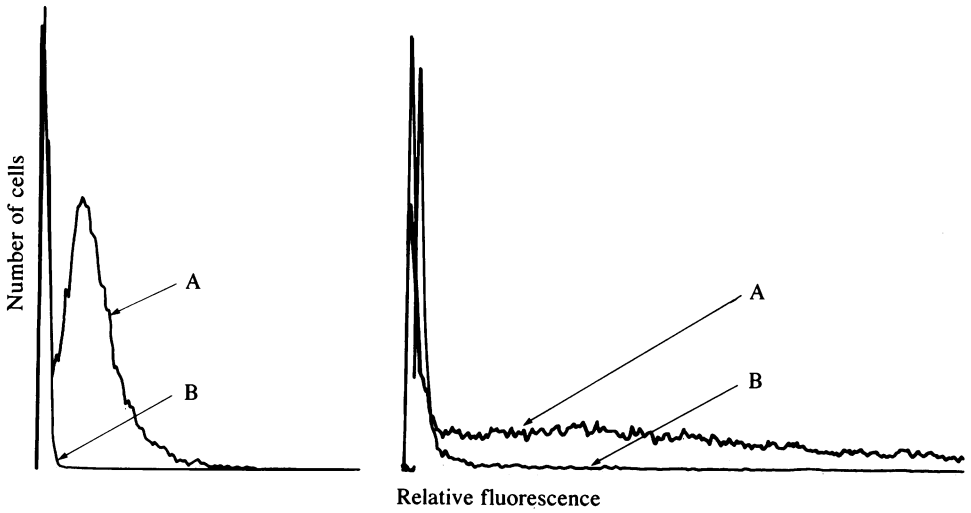

FIG. 1. Liposomes carrying the monoclonal antibody BE3 bind to T cells in PBL. (*Left*) BE3-PyL (trace A) or mIgG-PyL (trace B) (≈10 μg of IgG per ml and 10 μM pyrene) were incubated for 1.5 hr at 4°C with PBL at 10^7 cells per ml. The cells that had bound liposomes were assayed by indirect immunofluorescence [after the addition of goat anti-mouse $F(ab')_2$ fragments conjugated with fluorescein] using a flow cytofluorimeter. (*Right*) Liposomes [BE3-NBDL (trace A) and mIgG-NBDL (trace B)] similar to those in *Left* but containing NBD-Pam_2-PtdEtn, a fluorescent lipid (34), in place of the phototoxic lipid PyPam-PtdCho, were incubated with PBL under the conditions given in *Left*. Binding was assayed directly by flow cytofluorimetry, with the same settings as for fluorescein.

from unimmunized mice (mIgG-PyL) was attached did not bind to lymphocytes (Fig. 1). A further indication of the selectivity of binding was obtained after treating the lymphocytes with BE3 when the corresponding antigen is modulated (selectively lost) from the surface of the T cells (27, 35, 36). The modulated cells no longer bound BE3-PyL. The B-cell line GM 1056A also failed to bind these liposomes, whereas the T-cell line MOLT-4 expressed the BE3 antigen and bound BE3-PyL.

Phototoxic Liposomes Conjugated to BE3 Kill T Lymphocytes Upon Irradiation. We next tested the ability of BE3-PyL to inhibit the division of T lymphocytes after stimulation with the mitogen PHA. PBL in PBS were incubated with the liposomes. The cells were sequentially washed, irradiated at 320–400 nm, supplemented with culture medium, and stimulated with PHA. After 3 days the incorporation of [^{3}H]thymidine into DNA was assayed and found to be virtually absent (Fig. 2). The loss of ability to proliferate was paralleled by a dramatic decrease in the viability of the cells treated with BE3-PyL but not in those treated with mIgG-PyL (Fig. 3). The light dose of 8 J/cm^2 was the lowest level that reproducibly damaged the cells to a high extent after treatment with BE3-PyL.

In control experiments in which the PHA responses of PBL were measured, we found that BE3-PyL were without effect in the dark and that cells treated with PBS had the same response whether or not they had been irradiated with ultraviolet light. Liposomes carrying mouse IgG (mIgG-PyL) were slightly inhibitory upon ultraviolet-irradiation (Fig. 2), possibly because a small amount of PyPam-PtdCho is transferred to the cells through the aqueous phase (37) or because a small number of these liposomes bind to the cells, for example, through Fc receptors (38). Such binding was undetectable by cytofluorography, but the receptors involved might have been present in low numbers and particularly active in endocytosis.

When PBL were mixed with mIgG-PyL and irradiated without the washing step, the same PHA response was obtained as if the cells had been washed, implying that the toxic species formed on irradiation cannot diffuse from unbound liposomes to cells. This is the expected result when singlet O_2 is involved (39) because its mean diffusion path is about 200 nm in water (40).

Phototoxicity of Liposomes Conjugated to BE3 Is Selective. Three experiments were done to show that the damage brought about by the phototoxic liposomes was directed towards cells bearing the BE3 surface antigen. First, when the BE3 antigen was blocked with excess BE3, or when it was removed from the cell surface by modulation with excess BE3, T lymphocytes in PBL were no longer damaged by the phototoxic liposomes (Fig. 4).

Second, we examined the T/B cell ratio in PBL by cytofluorography 3 days after irradiation, using antigens that bind the monoclonal antibodies OKT3 and OKB2 (Ortho

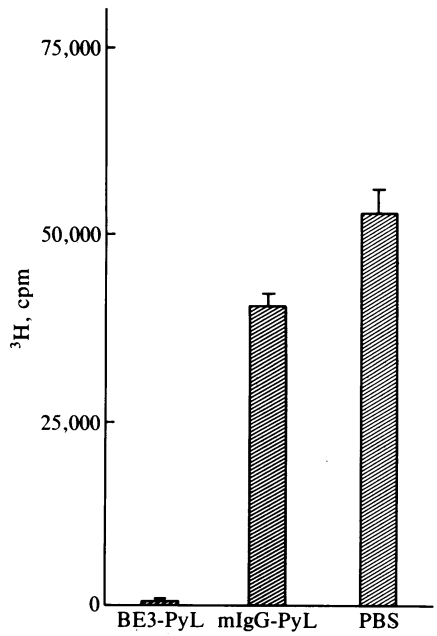

FIG. 2. Incorporation of [^{3}H]thymidine into the DNA of T cells in PBL after treatment with phototoxic liposomes, followed by stimulation with PHA. The cells were irradiated with ultraviolet light after incubation with BE3-PyL, mIgG-PyL, or PBS and were assayed as described. Each value shown is the average from four experiments, in each of which the PHA response was determined four or five times. The values are corrected for ^{3}H uptake in the absence of PHA (670 ± 350 cpm). Bars show standard deviations.

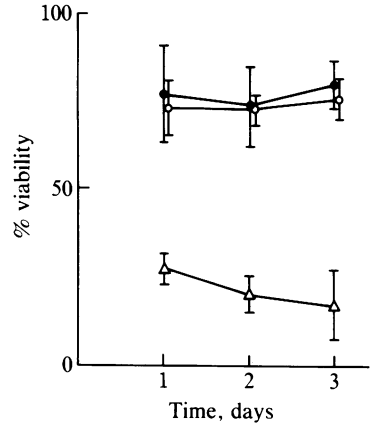

FIG. 3. Viabilities of PBL after treatment with phototoxic liposomes [BE3-PyL (△) or mIgG-PyL (○)] or PBS (●). The cells were from the experiments shown in Fig. 2 and were treated identically, excluding the stimulation with PHA. Viabilities were assayed by trypan blue exclusion, and the results were averaged as described in the legend to Fig. 2.

Diagnostics) as markers. The ratio (≈13:1) was unchanged in control experiments (irradiation with mIgG-PyL or PBS) but decreased to one-third (≈4.5:1) when the cells were irradiated after treatment with BE3-PyL. Initially the lymphocytes were 70% T cells and 6% B cells; therefore, at least 65% of the T cells must have been killed to account for the observed change in ratio. The percentage of affected cells is likely to be greater (compare the PHA response), as irreversibly damaged but morphologically intact cells would be counted in this experiment.

In the above experiment the integrity of the B cells was not assayed directly. Therefore, we carried out an experiment in which the effects of BE3-PyL on a B-cell line (GM 1056A) and a T-cell line (MOLT-4) were compared. The cells were treated with liposomes and irradiated as before. Spontaneous thymidine uptake over 24 hr was then measured. The B-cell line was hardly affected by the liposomes, while thymidine uptake by the T-cell line was largely eliminated (Table 1).

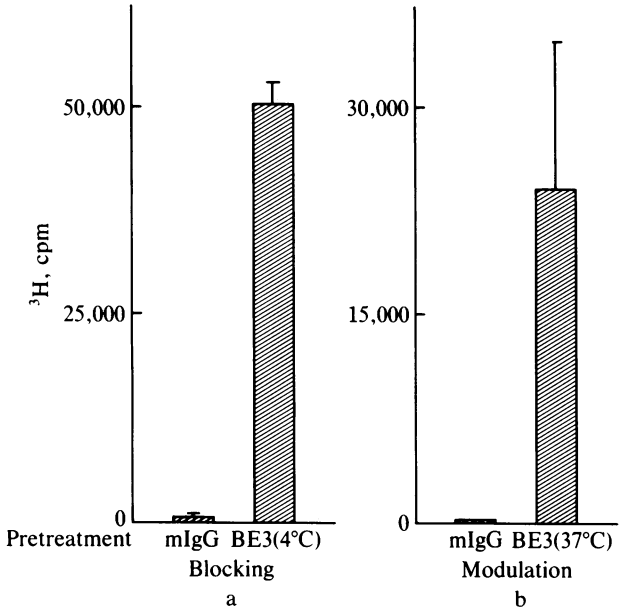

FIG. 4. Incorporation of [^{3}H]thymidine into the DNA of PHA-stimulated T cells in PBL after treatment with BE3-PyL under conditions where the BE3 antigen was blocked or absent. (*a*) The BE3 antigen was blocked by treating PBL with excess BE3 at 4°C. In a control experiment, cells were treated with mIgG [here: ascites fluid containing a mouse monoclonal IgG (γ1) that does not bind to lymphocytes]. (*b*) The BE3 antigen was modulated by prolonged treatment with excess BE3 at 37°C. A control sample was treated in parallel with mIgG. In a parallel experiment, the response of cells treated with mIgG-PyL was an uptake of 23,450 ± 10,000 cpm of [^{3}H]thymidine. In *a* and *b*, each value shown is the average of five determinations. Treatment with liposomes and irradiation were as described in Fig. 2 and *Materials and Methods*.

DISCUSSION

Two-fold specificity in drug delivery, which we have demonstrated here *in vitro*, has several advantages over other pharmacological procedures. Most important is the potentially high specificity. Even the most effective delivery system based on protein–drug conjugates cannot be absolutely specific (especially as the drug and its carrier will eventually become separated *in vivo*). For example, a large fraction of liposomes (but not all of them; ref. 41) are rapidly removed by the reticuloendothelial system. Even the majority of liposomes bearing cell-specific antibodies suffer this fate, and if a conventional cytotoxic drug were encapsulated within them, considerable damage to the liver, spleen, and bone marrow might be expected to occur (41, 42). In contrast, a phototoxic drug need only be activated at the desired site of action. In our case the toxic species (probably singlet O_2) is very short-lived and cannot even diffuse to neighboring cells.

A further advantage of the system we are using is that a high level of damage can be expected for each antibody molecule entering a cell. Each liposome carries ≈400 pyrenyl groups per attached antibody, and each of the pyrenyl groups can catalyze the formation of numerous singlet O_2 molecules. One mechanism by which the phototoxic liposomes might act is by becoming incorporated into lysosomes after uptake by endocytosis (43), damaging the membranes of these organelles, and thereby causing the release of degradative enzymes into the cytoplasm (15, 44). With such an amplification mechanism, very few antibody molecules need be introduced into the target cells compared with other drug delivery systems, except those that use toxins with catalytic activities.

The system is also versatile; both hydrophilic and hydrophobic drugs may be used in combination with a variety of antibodies or hormones. A lipid bilayer has a surprisingly high capacity for carrying drug molecules, and because pyrene is a hydrophobic molecule, it was incorporated into the bilayer of the liposomes rather than trapped in the interior, as is commonly done with hydrophilic drugs such as methotrexate (1, 3, 4).

Because BE3-PyL selectively affect T lymphocytes, BE3-PyL and liposomes carrying antibodies against subsets of T lymphocytes should be tested in an extracorporeal device in which blood is transiently exposed to ultraviolet radiation. Such a device has already been used to treat patients with simple photoactivatable drugs such as psoralens (45). In this manner it may be possible to selectively deplete subpopulations of T lymphocytes involved in immunologic diseases, including malignancies, autoimmune diseases, and immune deficiencies. In the long term, such highly specific drugs

Table 1. [^{3}H]Thymidine uptake by a B-cell line and a T-cell line after treatment with BE3-PyL*

Treatment	Cell line	
	GM 1056A (B cells)	MOLT-4 (T cells)
PBS (control)	135,879 ± 3302	339,328 ± 20,987
BE3-PyL	123,461 ± 5840	2,926 ± 256

*This experiment was performed with 100-nm liposomes containing the lipid $PyPam_2$-PtdEtn. The values given are the averages of five determinations with their standard deviations.

might be useful for treatment of solid tumors, particularly micrometastases, using fiber optics (16). For this purpose, smaller carriers capable of crossing the microvasculature should be investigated (4).

This work was supported by a grant from Johnson & Johnson Cardiovascular Inc.

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