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Antibody-targeted photolysis: Selective photodestruction of human T-cell leukemia cells using monoclonal antibody-chlorin e_6 conjugates

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ABSTRACT **Selective *in vitro* photodestruction of HPB-ALL human T-cell leukemia cells was accomplished using the photosensitizer chlorin e_6 coupled through dextran molecules to an anti-T-cell monoclonal antibody (mAb), anti-Leu-1. Conjugates with mAb/chlorin molar ratios as high as 1:36 retained mAb binding activity, and the absorption spectrum and quantum efficiency for singlet oxygen production of bound chlorin (0.7 ± 0.2) were unchanged from that of the free photosensitizer. Phototoxicity, as measured by a clonogenic assay and by uptake of ethidium bromide, was dependent on the doses of both mAb–chlorin and 630- to 670-nm light, was enhanced by ^2H_2O, and was observed only in target populations that bound the mAb. Similarly, free chlorin e_6 in solution had no photodynamic effect in amounts 100 times more than that carried by the mAb. For this antibody-targeted system, approximately 10^{10} molecules of singlet oxygen were necessary to kill a cell.**

A principal objective in the development of chemotherapeutic strategies is to devise modalities that are capable of selectively acting on the offending cells while sparing normal tissues. Selectivity may be provided by the preferential action of a particular agent or by preferential localization of molecules that act nonspecifically. Monoclonal antibodies (mAbs) that bind to cell surface antigens have great promise for directing cytotoxic agents to appropriate sites; and a substantive body of literature has already accumulated on the use of monoclonal antibodies to enhance selectivity of cytotoxic drugs, radioisotopes, or toxins (1–4).

The selectivity of mAb-directed treatment may be reduced, however, by cross-reactivity due to shared antigens on normal tissues and by nonspecific tissue uptake (5, 6). These problems can be obviated by utilizing mAbs that carry light-activated molecules (i.e., photosensitizers, PSs), which are innocuous without illumination but which produce toxic or reactive species such as singlet oxygen ($^1O_2$) upon absorbing light. By confining illumination to areas containing specifically bound PSs, toxicity can be limited to target cells. Nontarget cells, which adventitiously bind the mAb-PS but which are outside the illuminated volumes, would be spared.

The present report describes selective cytolysis of human T-cell leukemia cells *in vitro* using mAb conjugated to the PS chlorin e_6, which has a molar extinction coefficient of about 59,000 $M^{-1}\cdot cm^{-1}$ at 660 nm (7). Chlorin/mAb conjugation ratios of approximately 30:1 were achieved, and mAb and chlorin activities were retained. Selective destruction of target T cells was accomplished by irradiation of cells containing mAb-PS conjugates bound to their cell surface.

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MATERIALS AND METHODS

Chromophore Preparation. In brief, chlorin e_6-monoethylenediamine monoamide (chlorin e_6-A) was prepared as follows: A monoactivated species (mixed anhydride) was formed by sequential addition of triethylamine and ethyl chloroformate to chlorin e_6 dissolved in anhydrous *N,N'*-dimethylformamide. TLC analysis using C_{18}-bonded silica plates in MeOH/0.01 M sodium phosphate, 7:3 (vol/vol), pH 6.85, showed 80–90% monoactivated chlorin e_6, with unreacted chlorin e_6 second in abundance and with only a trace of the diactivated species.

A 10-fold molar excess of ethylenediamine was then added to the monoactivated chlorin. After 15 min, TLC analysis showed a new pigment, with intermediate mobility and with an abundance estimated at 80%. The reaction mixture was diluted with distilled water and applied to a C_{18} reverse-phase column. Elution with 35% (vol/vol) methanol in 0.1 M sodium phosphate, pH 6.85, was used to remove residual chlorin e_6 and to convert the remaining carboxyl groups to the sodium salt form. A step gradient of methanol in H_2O [45% followed by 55% (vol/vol)] was used to elute the ethylenediamine derivative. The preparation was shown to be homogeneous by reverse-phase TLC.

mAb Purification. An anti-T-cell mAb anti-Leu-1 (L17-F12, a kind gift of R. Levy, Stanford University Medical Center), and an anti-bovine serum albumin (BSA) mAb, 9.1, were used. The anti-Leu-1 mAb in ascites fluid was precipitated twice with ammonium sulfate, followed by chromatography on Sephadex-G-200 (11). The anti-BSA mAb was purified in two-step procedure by affinity chromatography on a BSA-Sepharose 4B column, followed by gel permeation chromatography on a Sephadex G-200 column (11). mAb purity was checked by cellulose acetate and $NaDodSO_4$/polyacrylamide gel electrophoresis, and isoelectric focusing.

Radioiodination of mAb. mAb preparations were radiolabeled with ^{125}I by the iodine monochloride method (8). Radiolabeled preparations were chromatographed on Sephadex G-200, to remove any aggregated material generated by the labeling process. Specific activities of 0.12–0.20 mCi/mg (1 Ci = 37 GBq) were obtained, and >99% of the radioactivity was precipitable by 10% (wt/vol) trichloroacetic acid.

Preparation of mAb-Chlorin Conjugates. Conjugates were prepared by first coupling the chlorin to a dextran polymer (9) and, subsequently, binding the polymer-PS to the mAb via its carbohydrate moiety. Dextran (10 kDa) was oxidized with 2 mM sodium metaperiodate for 1 hr at room temperature. The reaction mixture then was applied to a Sephadex G-25

Abbreviations: mAb, monoclonal antibody; PS, photosensitizer; BSA, bovine serum albumin; GAM-FITC, fluoresceinated goat anti-mouse IgG; EtdBr, ethidium bromide; ATPL, antibody-targeted photolysis.

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column to remove excess periodate and was lyophilized. The oxidized dextran was reacted with a 5-fold molar excess of ethylenediamine, followed by the addition of 75-fold molar excess of chlorin e_6-A. Ethylenediamine was included to provide an amine functionality on the dextran for subsequent coupling to the mAb. After agitation at 4°C for 12 hr, the imine bonds formed were reduced by a 2-hr incubation with 100-fold molar excess of sodium borohydride. The dextran-chlorin conjugate was chromatographed twice on a Sephadex G-50 column, desalted on a G-25 column, eluted from a reverse-phase column with 50% (vol/vol) methanol in H_2O, and lyophilized.

Oxidation of the mAb carbohydrate was performed as described (10). Oxidized mAb was reacted with a 125-fold molar excess of chlorin-dextran conjugate, and the resulting conjugate was reduced by incubation with a 140-fold molar excess of sodium cyanoborohydride. The mAb-dextran-chlorin conjugate was purified on a Sephacryl S-300 column (100 × 2.6 cm) equilibrated with 0.1 M sodium phosphate, pH 6.5.

Characterization of Conjugates. mAb and conjugate activities were measured using radiobinding assays (12). mAb/PS substitution ratios were determined using radioiodine quantitation for the mAb concentrations and absorbance at 400 and 660 nm for the chlorin concentration.

The effect of conjugation on the binding of polyclonal, fluoresceinated goat anti-mouse IgG (GAM-FITC) (Tago, Burlingame, CA) was determined by fluorescence microscopy and fluorescence-activated cell sorter analysis. In brief, for the microscopy, cells were incubated with saturating amounts of conjugated or unconjugated mAb for 30 min at 4°C, washed, reincubated with GAM-FITC, washed, and examined with a Zeiss fluorescence microscope—100–200 cells were counted in multiple fields.

For the fluorescence-activated cell sorter study, aliquots of HPB-ALL cells were sequentially incubated with multiple concentrations of unconjugated and conjugated antibody, and then with an excess of GAM-FITC. The maximum median channel fluorescence (i.e., when all mAb binding sites were occupied) and the mAb concentrations necessary to give 50% of this fluorescence were determined.

Photochemical Characterization. Singlet oxygen yield was determined by measuring the destruction of *N,N*-dimethyl-4-nitrosoaniline in the presence of histidine (13) using rose bengal as a standard (14). In brief, samples of rose bengal, chlorin e_6, or chlorin-mAb conjugates were mixed with 0.01 M histidine and approximately 2×10^{-5} M *N,N*-dimethyl-4-nitrosoaniline in air-saturated Dulbecco's phosphate-buffered saline (PBS, GIBCO) and irradiated. Photostability was determined by measuring the change in both the Soret band and the 660-nm absorption band with 630- to 670-nm irradiation of the chlorin preparations.

***In Vitro* Photolysis.** HPB-ALL T-leukemia cells or control cells (either the SB human B lymphoblastoid cell line or CV-1 monkey kidney cells), which do not express the Leu-1 antigen, were grown in RPMI 1640 (GIBCO) supplemented with 10% (vol/vol) fetal calf serum/1% penicillin/1% streptomycin (complete medium). In experiments using anti-BSA conjugates, cells were grown in media with horse serum rather than fetal bovine serum. Before each experiment viability was determined using ethidium bromide (EtdBr) staining and fluorescence microscopy. When necessary, the cells were centrifuged in a Ficoll/Hypaque gradient so that initial viability was >90–95%.

The cells were incubated with conjugate for 30 min at 4°C, washed twice with cold PBS, and irradiated with the xenon source filtered to deliver light between 630 and 670 nm. The dose rate was typically 0.6 mW/cm^2. Controls included unirradiated cells and cells exposed to light alone, as well as irradiated and unirradiated cells that had been treated with unconjugated anti-Leu-1, irrelevant (anti-BSA) mAb-conjugates, or free chlorin e_6 in amounts 100-fold more than that delivered by the conjugates. Experiments investigating the effect of ^2H_2O were carried out as above, except that the cells were irradiated in buffer made by diluting 10× PBS with ^2H_2O.

After irradiation, the cells were washed, and complete medium was added. Aliquots were taken, cells were counted, and immediate and delayed phototoxicity was assayed using EtdBr staining and fluorescence microscopy. To enumerate cell survival, the cells were seeded into 24-well plates using a two-layer (0.25%/0.5%) soft agarose system (SeaPlaque agarose, FMC, Rockland, ME) with a feeder layer of 3×10^5 HPB-ALL cells per well. After approximately 2 weeks, colonies were counted using a stereomicroscope.

RESULTS

Chromophore Preparation. Chlorin e_6-A was synthesized as described above. Verification of the monosubstitution was accomplished by TLC and elemental analysis. Reverse-phase TLC produced a single spot, and elemental analysis of a sample crystallized from ethyl acetate as the monohydrochloride gave the following values: $C_{36}H_{43}N_6O_5Cl$ (675.22): C, 63.82%; H, 6.69%; N, 11.46%. Expected values were as follows: C, 64%; H, 6%; N, 12.45%. The presence of a free primary amine was also indicated by the compound's solubility at the isoelectric point (about pH 3) at which underivatized chlorin e_6 aggregates and precipitates from solution. The absorption spectrum of chlorin e_6-A was identical to the parent molecule chlorin e_6 (Fig. 1*A*).

Conjugate Characterization. Anti-Leu-1 and anti-BSA conjugates were prepared and characterized as to binding properties, chromophore substitution ratios, and photochemical activity. The conjugates were isolated on Sephacryl S-300 as a broad peak corresponding to an average molecular weight of 210,000. As shown in Table 1, conjugate preparations retained most of their original activity, as demonstrated by binding to either T cells or immobilized BSA. Substitution ratios of 24–36 chromophores per mAb were achieved. Attachment of chlorin to mAb caused no significant changes in the chlorin absorption spectrum (Fig. 1*B*).

The coupling process did not completely block the Fc portion of the mAb, since anti-Leu-1 chlorin conjugates bound to T cells could be detected using GAM-FITC. Fluorescence-activated cell sorter analysis comparing HPB-ALL cells stained with anti-Leu-1-chlorin–GAM-FITC and unconjugated anti-Leu-1–GAM-FITC showed 42% less median channel fluorescence of the conjugate, relative to the unconjugated mAb. Conjugate activity, based on the concentration necessary to achieve 50% saturation of binding sites, was about 80% of the unconjugated mAb, in agreement with the results using radiolabeled mAbs (Table 1). By fluorescence microscopy >99% of the HPB-ALL cells stained with anti-Leu-1-chlorin conjugate and GAM-FITC, while <5% of CV-1 or human SB cells were stained. Anti-BSA-chlorin stained <5% of any cell line tested. Thus conjugation preserved mAb binding specificity.

Singlet oxygen production was determined for chlorin e_6 and the mAb-chlorin conjugate relative to a rose bengal standard (13). As shown in Table 2, the quantum yield for $^1O_2$ production was 0.7 ± 0.2 for both the free chlorin and the conjugate. Therefore, coupling did not alter the capacity for $^1O_2$ production. The photostability was also unaffected. For both chlorin and conjugate, there was <20% bleaching (as determined by loss of the Soret band or the 660-nm absorption) after 50 J/cm^2 irradiation (630- to 670-nm bandpass).

***In Vitro* Photolysis.** The efficacy of antibody-targeted photolysis was examined by incubating target or control cells with saturating concentrations of conjugate, washing to

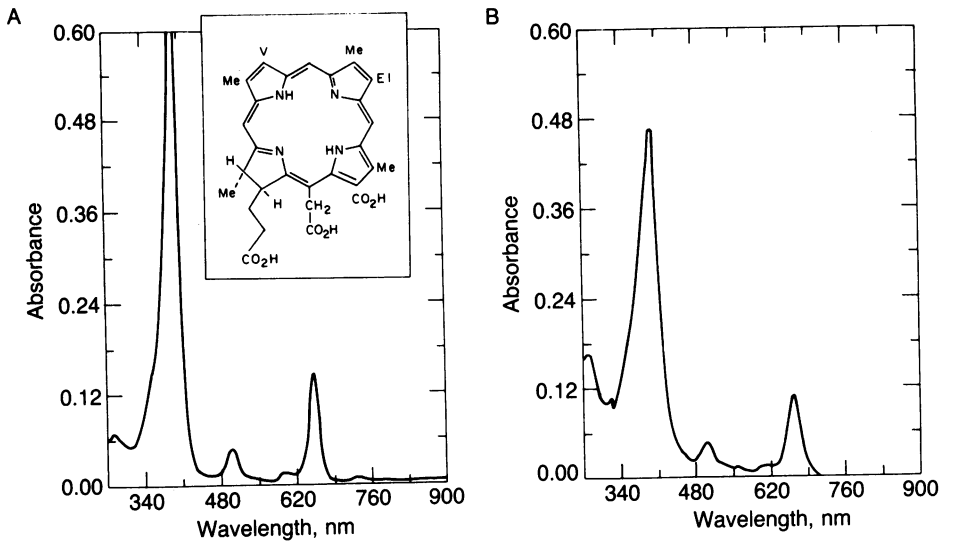

FIG. 1. (*A*) Overlapping absorption spectra of chlorin e_6 and chlorin e_6-monoethylene diamine are shown. *Inset* shows the structure of the PS. (*B*) Absorption spectrum of an anti-Leu-1-dextran-chlorin e_6 conjugate is shown.

remove unbound material, and subjecting the cells to increasing doses of 630- to 670-nm light from a filtered xenon arc source. To avoid antigenic modulation by the bound anti-Leu-1, incubation and irradiation were performed at 4°C. After irradiation, cell number and viability (by EtdBr exclusion) were ascertained, and cells were cloned into soft agarose or replaced in culture. Both clonogenic and EtdBr exclusion assays were used; the former was used to assess reproductive potential, and the latter was used to provide a measure of membrane integrity.

The anti-Leu-1-chlorin conjugates caused selective, light-dependent cell killing of HPB-ALL cells. A typical survival curve for a conjugate preparation with chlorin/mAb molar ratio of 31:1 is shown in Fig. 2. About 58 J/cm^2 was necessary to kill 90% of the T cells. This dose of light alone caused about a 7% decrease in surviving fraction. The anti-BSA-chlorin conjugates had <5% effect on the T cells, while the anti-Leu-1 conjugate had a similarly small effect on CV-1 monkey kidney cells or on SB human B lymphoblastoid cells that do not bind the mAb.

By EtdBr exclusion, there was no change in the number of "viable" cells immediately after irradiation. The fraction of EtdBr-positive cells increased with time and reached a plateau after about 15–18 hr (data not shown). Typical phototoxicity curves measured 18 hr after irradiation are shown in Fig. 2*B*. Two different anti-Leu-1-chlorin preparations with chlorin/mAb molar ratios of 28:1 and 31:1 were both phototoxic to target HBP-ALL cells. As in the case of the clonogenic assay, about 50 J/cm^2 were necessary to cause membrane damage to 90% of the T cells. Similarly, anti-BSA-chlorin conjugates had no effect on HPB-ALL cells, and the anti-Leu-1-chlorin preparations had no effect on CV-1 or on SB cells (Fig. 2*B*). Light alone had <5% effect on EtdBr uptake in any of the cells (data not shown).

Table 1. Characterization of mAb-chlorin conjugates

Sample	Antibody	Maximum % binding*	mAb/Chromophore molar ratio
1	Anti-Leu (unconjugated)	95	—
2	Anti-Leu-1-chlorin	73	1:30
3	Anti-BSA (unconjugated)	93	—
4	Anti-BSA-chlorin	76	1:24
5	Anti-BSA-chlorin	71	1:31
6	Anti-BSA-chlorin	74	1:36

*Binding of radiolabeled mAb or conjugate to T cells or BSA-Sepharose. Thyroglobulin (0.5%) was used in the assay to prevent nonspecific adsorption.

Some experiments were carried out in 90% ^2H_2O, which increases the lifetime of singlet oxygen and enhances damage due to this species (15). Phototoxicity was increased in ^2H_2O (Table 3), and the light dose necessary to cause 90% phototoxicity was decreased by about a factor of 2.

There are approximately 5×10^5 anti-Leu-1 binding sites on HPB-ALL cells (16). Therefore, when all sites are occupied by conjugate, about 3×10^{-15} g of chlorin e_6 is localized at the surface of each cell. In experiments testing the effect of free chlorin, 10^5 cells were incubated with 10^{-10} to 10^{-8} gm of chlorin e_6 for 30 min at 4°C, washed, and irradiated with 80 J/cm^2 of 630- to 670-nm light. No effect on EtdBr uptake was observed (Fig. 2*B*).

To investigate the effect of conjugate dose on membrane phototoxicity, aliquots of 6×10^4 HPB-ALL cells were incubated with doses of anti-Leu-1-chlorin from 0.001 to 1 μg, washed, and irradiated with 80 J/cm^2 light, as above. The average results from two experiments demonstrate that EtdBr uptake depends on the amount of conjugate used (Fig. 3). There was a parallel relationship between the doses of light and of conjugate necessary to produce comparable phototoxicity. Thus, with a saturating amount of conjugate, a decrease from 80% to 90% EtdBr excluding cells required a 19-fold increase in fluence (Fig. 2). Using a constant, 80 J/cm^2 fluence, an 18-fold increase in conjugate was necessary to cause the same change in EtdBr exclusion (Fig. 3).

DISCUSSION

Chemotherapeutic strategies based on preferential localization of PS molecules offer 2-fold selectivity: from targeting of the PS and from spatial control of the irradiation. With antibody-targeted photolysis (ATPL), because the mAb is responsible for the targeting, the PS need have no intrinsic tumor localizing properties, but can be optimized for spectral and photosensitizing properties. To permit *in vivo* applications, the PS should have substantial long wavelength absorbance above 600 nm, so that incident light is not absorbed by tissue chromophores such as hemoglobin (17). The conjugate coupling procedure must allow for high PS/mAb molar substitution ratios while preserving the activity of both components.

Table 2. Single oxygen production

Chromophore	ϕ ($^1O_2$), mol
Rose bengal	0.72 (ref. 14)
Chlorin e_6	0.7 ± 0.2
Anti-Leu-1-chlorin e_6	0.7 ± 0.2

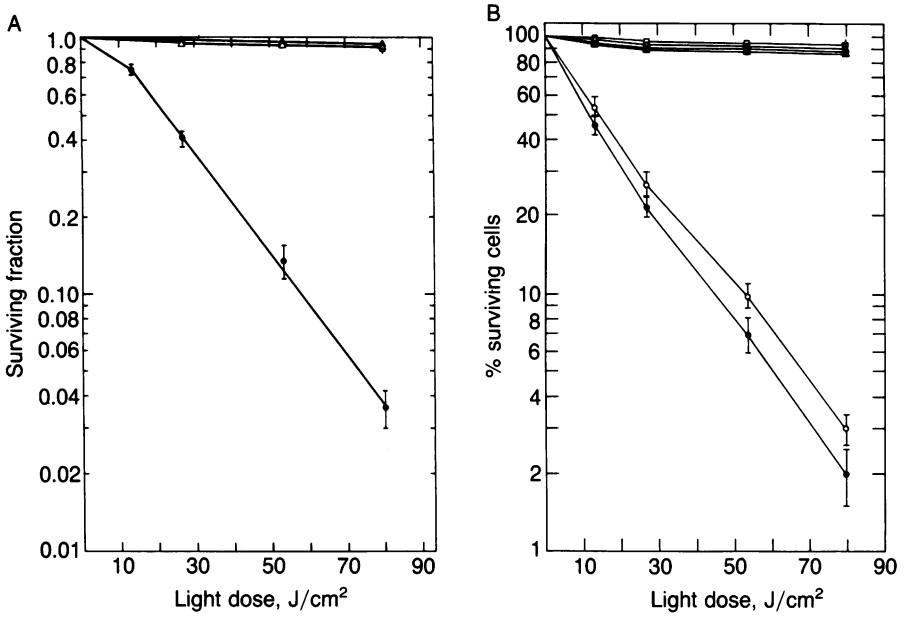

FIG. 2. (*A*) Surviving fraction of cells, after treatment with saturating amounts of mAb-chlorin e_6 conjugates, is shown. The data are the average of two experiments (mean ± SD), each including six replicates for each light dose. ●, HPB-ALL cells/anti-Leu-1-chlorin e_6 (mAb/chlorine molar ratio 1:31); △, HPB-ALL cells/anti-BSA-chlorin e_6; +, HPB-ALL cells/light; ▲, SB cells/anti-Leu-1-chlorin e_6. (*B*) Percent of cells excluding EtdBr ("surviving cells") 18 hr after treatment with saturating amounts of mAb-chlorin e_6 conjugates or free chlorin e_6 and light is shown. The data are the average of two determinations (mean ± SD). ○, HPB-ALL cells/anti-Leu-1-chlorin e_6 (1:28; mAb/chlorin molar conjugation ratio); ●, HPB-ALL cells/anti-Leu-1-chlorin e_6 (1:31; mAb/chlorine molar ratio); △, HPB-ALL cells/anti-BSA-chlorin e_6; +, HPB-ALL cells/free chlorin e_6; ▲, SB cells/anti-Leu-1-chlorin e_6; □, CV-1 cells/anti-Leu-1-chlorin e_6.

This report documents studies on selective killing of HPB-ALL human T-leukemia cells using chlorin e_6 coupled with dextran carriers to an anti-T-cell mAb. Chlorin e_6 is photostable, and it is nontoxic in mice at doses up to 100 mg/kg (J.C.B., unpublished data). The chlorins are effective PSs (Table 2, refs. 18 and 19), with significant long wavelength absorption; the extinction coefficient of chlorin e_6 being about 59,000 M^{-1} cm^{-1} at 660 nm (7). By comparison, the more commonly employed PS hematoporphyrin derivative (HPD) has a substantially lower absorbance; the extinction coefficient hematoporphyrin monomer is only about 4300 M^{-1} cm^{-1} near 630 nm (20). The more substantial absorbance of chlorin e_6 is an advantage for a mAb-directed PS, since relatively small quantities of PS are localized at the cell surface.

Chlorin e_6 was modified by converting one of its carboxyl groups to an amino functionality by condensation with ethylenediamine. On the basis of ^{13}C NMR data obtained from other monoamides prepared from chlorin e_6, it is very likely that the compound is a mixture of isomers with the derivatization at either the acetic or the propionic group of the chlorin (J.C.B., unpublished results). However, the elemental analysis indicates that no more than one ethylenediamine moiety is present in each molecule.

We have shown (20) that direct attachment of hematoporphyrin molecules to the carbohydrate moieties on the Fc portion of both anti-Leu-1 and other mAbs protected the antigen binding site and permitted highly selective phototoxicity. With direct attachment, however, less than five chromophores could be directly linked to the mAb (21). To increase this ratio, dextran-dye conjugates were prepared and linked to the mAb carbohydrate. The MAb-chlorin conjugates retained binding specificity and were selectively phototoxic, only killing cells that bound the mAb. In the absence of light, specifically bound conjugates had no appreciable toxicity. With illumination, the conjugates effectively produced $^1O_2$ (Table 2). The enhancement of cellular phototoxicity in ^2H_2O (Table 3) suggests that diffusible $^1O_2$ is at least partially responsible for the light-induced damage.

Phototoxicity depended in a comparable manner on both incident light and amount of conjugate. This is reasonable, because production of $^1O_2$ depends on absorbed photons, which is the product of incident light dose and number of absorbing molecules. Because only small amounts of chlorin (10^{-15} g) were bound to each cell, phototoxic light doses were correspondingly large, at 80 J/cm² to kill 90% of the cells. However, this amount of light alone had no significant effect on the cells. An increase in the number of bound mAbs should reduce the photolytic light dose. mAb-conjugate binding and irradiation were carried out at 4°C, so there should have been no internalization of the membrane-bound conjugates. Thus it is not necessary for the chlorin to enter the cells to induce

Table 3. Enhancement of anti-Leu-1-chlorin phototoxicity with ^2H_2O

Light dose, J/cm²	Phototoxicity	
	PBS	^2H_2O
0	98 ± 2	90 ± 4
13.3	59 ± 3	34 ± 4
26.6	37 ± 4	9 ± 5

Phototoxicity was measured as the fraction of HPB-ALL cells (mean ± SD, $n = 4$) excluding EtdBr, assayed 24 hr after irradiation. Light alone (26.6 J/cm²) caused 3% EtdBr incorporation.

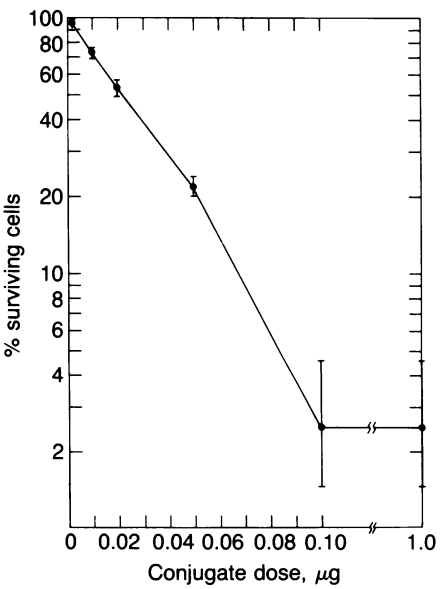

FIG. 3. Percent of HPB-ALL cells (mean ± SD) excluding EtdBr ("surviving cells") 18 hr after treatment with various amounts of anti-Leu-1-chlorin e_6 conjugate and 80 J/cm² of light. The data are the average of two experiments.

photolysis. Unconjugated chlorin under the same conditions, however, was not phototoxic.

Although the site(s) for mAb-localized $^1O_2$ damage have not yet been elucidated, the typical 0.1- to 0.2-μm diffusion distance of $^1O_2$ (22) suggests that the cell membrane may be a principal target. In addition, cytoplasmic constituents near the membrane might also be affected. The damage process did not immediately alter membrane permeability, since the cells continued to exclude EtdBr at the end of the irradiation and for some hours afterwards. For many chemotherapeutic agents, dye exclusion assays have been found to significantly under-report reproductive potential and "viability" (23, 24). In contrast, our results show that EtdBr exclusion is at least as sensitive as the clonogenic assay. This correlation seems reasonable if ATPL primarily damages the plasma membrane, because EtdBr exclusion is a measure of membrane integrity.

Since the number of mAbs bound at the cell surface is known (15), it is possible to roughly quantify the photolytic process. The molecular cross section σ for absorption of photons is 2300 ε/N_A cm^2, where ε is the molar extinction coefficient and N_A, Avogadro's number, is 6×10^{23} molecules per mol (25). For chlorin e_6, $\sigma = 2 \times 10^{-16}$ cm^2. The number of incident photons per cm^2, n_{pi}, for an incident fluence of 50 J/cm^2 at 660 nm is 1.7×10^{20} photons per cm^2, since the photon energy is hc/λ (h, 6.6×10^{-34} J·sec; and c, 3×10^{10} cm/sec). The number of absorbed photons per cell, n_{pa}, is given by $n_{pi}\ \sigma\ n_c$, where $n_c = 1.5 \times 10^6$ is the number of chlorins per cell. Thus, neglecting photodestruction of the chlorins, about 5×10^{10} photons are absorbed per cell killed, and about 3×10^{10} molecules of $^1O_2$ are produced (see Table 2). Since half of the $^1O_2$ diffuses away from the cell surface, an upper limit of approximately 10^{10} molecules of $^1O_2$ are necessary to kill a cell.

Similar estimates of n_{pa} can be made for HPD, using uptake and fluence data reported for *in vitro* phototoxicity (26). For example, Bellnier and Dougherty (26) used about 2.3×10^{-13} g of HPD per cell and a light dose of 2.2 J/cm^2 (590–650 nm) to kill 90% of the cells. Using the above analysis and taking an estimate of $\varepsilon = 4300\ M^{-1}\ cm^{-1}$ for HPD, on the order of 10^{10} photons are absorbed per cell killed with HPD, which is comparable to the present work. This correspondence must be interpreted with caution, however, since the intracellular extinction coefficient(s) of the active component(s) of HPD are not certain, and the location and sensitivity of the sites of HPD photodamage [e.g., mitochondria (27)] may be quite different from the perimembrane damage sites for ATPL. In any case, 200–1000 times more PS per cell is typically used for the HPD studies (26–28) compared to the mAb-chlorin.

Mew *et al.* (29, 30) demonstrated selective mAb-directed phototoxicity *in vitro* and *in vivo* using hematoporphyrin attached directly to anti-tumor antibodies with carbodiimide. Their reported hematoporphyrin/mAb conjugation ratios of $\geq$60:1 are much greater than what has been generally reported for carbodiimide-mediated coupling of small molecules to amino groups on mAbs (31, 32) and might be due in part to hematoporphyrin and hematoporphyrin-aggregate adsorption onto the mAbs. Neither the binding activities of their conjugates nor the antigen densities of their target cells were reported, so that comparisons with their work are difficult to make. Nonetheless, their success in treating murine tumors (29) indicate that ATPL can be effective *in vivo* and suggest that it may be possible to administer significantly less PS than is typically used with HPD. The lower dose and potentially more selective binding may reduce the cutaneous phototoxicity that is a common complication of HPD therapy.

ATPL also may have several advantages compared with mAbs used alone or as carriers of radioisotopes, drugs, or toxins. First, because of the dual selectivity involved (mAb binding and illumination), the mAb need not be completely tumor selective. As long as the cross-reactive or nonspecifically bound mAb-PS is not illuminated, there will be no adverse effects. Second, the mAb need have no intrinsic biological effector activity, since it is used only as a carrier. Third, in contrast to most drugs and toxins, the mAb-PS need not be internalized. Because of the diffusion distance of singlet oxygen, even mAb-PS-antigen complexes that are shed but remain near the tumor cells may be effective. And last, because of $^1O_2$ diffusion, antigen-negative tumor cells adjacent to cells that bind the mAb-PS may still be killed. However, the *in vivo* behavior of the mAb-PS conjugates, including pharmacokinetics and biodistribution, still needs to be established.

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