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Antibody-targeted photolysis: *In vitro* studies with Sn(IV) chlorin e6 covalently bound to monoclonal antibodies using a modified dextran carrier

(immunoconjugates/antibody modification/photosensitization/tumor cells)

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ABSTRACT **A monoclonal antibody-dextran-Sn(IV) chlorin e6 immunoconjugate was prepared by a technique involving the site-specific covalent modification of the monoclonal antibody oligosaccharide moiety. Dextran carriers were synthesized with a single chain-terminal hydrazide group, which was used as the coupling point between the carrier and the monoclonal antibody carbohydrate. Selective *in vitro* photolysis of SK-MEL-2 human malignant melanoma cells was accomplished using several conjugates prepared from anti-melanoma 2.1 (chromophore:antibody molar ratios, 6.8 and 11.2). Phototoxicity, as measured by clonogenic assay, was dependent on the delivered dose of 634-nm light and was observed only for conjugates that bound SK-MEL-2 cells. As judged by competitive inhibition radioimmunoassay, conjugates prepared in this fashion showed excellent retention of antigen binding activity relative to the unmodified antibody.**

Over the past decade, advances in monoclonal antibody (mAb) technology (1) have provided the opportunity to study the potential of mAbs to selectively deliver a variety of active agents (e.g., drugs, toxins, radioisotopes) to cell surfaces (2–8). The selectivity of such conjugates can be compromised by shared antigen cross-reactivity or by nonspecific uptake of the conjugates (9, 10). By employing photosensitizer (PS) active agents, these potential drawbacks are minimized as a result of the double selectivity required (i.e., antigen binding coupled with light activation of the PS). Although extensive work with PS alone has been reported (11, 12), comparatively few studies on mAb·PS immunoconjugates have been published (13, 14).

Reports on the use of mAb·PS conjugates have highlighted the need for a general method for producing active, chemically defined immunoconjugates. Most direct coupling procedures have modified amino acid side chains (13, 15) and produced structurally heterogenous populations of immunoconjugates with a range of binding properties (16). Efforts to direct the PS to a particular site on the mAb (e.g., carbohydrate) have produced more homogenous conjugates but appear limited in the number of PSs that can be coupled to a single mAb. An additional feature of previous conjugation schemes is that a significant fraction of the PS is physically adsorbed and not covalently bound to the mAb.

The present report documents the development of a reproducible technique for the assembly of chemically defined active immunoconjugates containing the PS Sn(IV) chlorin e6 (SnCe6). In the present technique, multiple PSs are covalently linked to the mAb through an end-functionalized dextran carrier. The carrier polymer method allows for preparation of reproducible mAb·PS conjugates with a range of PS-mAb molar ratios and generalization to other active agents (e.g., drugs, toxins, radioisotopes). Anti-melanoma·SnCe6 immunoconjugates prepared in this fashion were shown to be immunologically active and effective in selectively killing SK-MEL-2 human malignant melanoma cells.

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MATERIALS AND METHODS

Synthesis of SnCe6-Dextran Carrier. The synthetic schemes used in the current work are outlined in Fig. 1 (17). Dextran (Pharmacia T-40; weight average molecular weight ≈35,000) was chosen as the carrier polymer because it is water soluble, is nontoxic, and possesses a single reducing terminus. A reactive hydrazide group was added to the reducing terminus via reductive amination with a large excess of apidic dihydrazide in the presence of sodium cyanoborohydride (18, 19). Titration of the reducing end in I using the dinitrosalicylate method (20) showed a 92% modification of the terminal aldehyde relative to unaltered dextran. To prevent modification of the chain-terminal hydrazide in I prior to linkage to the antibody, a Trt protection group was introduced via the active ester TCPPH to yield II. In addition to providing a protected terminal hydrazide, the TCPPH was also designed to introduce a six-carbon spacer group on the dextran terminus in order to facilitate subsequent linkage to the mAb. The presence of the terminal Trt protection group was verified by visible spectroscopy of the Trt cation (λ_{max} = 430 nm) following digestion of II with 60% $HClO_4$ (21).

The terminally protected trityl-hydrazidodextran (II) was allowed to react with ethyl chloroformate in dimethyl sulfoxide DMSO containing triethylamine to yield the dextran-*trans*-carbonate (22). The isolated dextran-*trans*-carbonate was subjected to solvolysis with hydrazine hydrate to produce the dextran carbazate (III). SnCe6, prepared using the general method of Falk (23), was coupled to III via a hydroxybenzotriazole (HOBt) active ester of SnCe6 (SnCe6-HOBt) generated *in situ* in a separate vessel. The SnCe6 was trace-labeled with ^{113}Sn prior to coupling to III to facilitate quantitation. Following purification of the terminally protected SnCe6-dextran carrier on Sephadex G-50, side chain hydrazides, which had not reacted with SnCe6-HOBt, were capped by reductive amination with acetaldehyde in 0.3 M acetate buffer (pH 5.0) containing 200 mM $NaCNBH_3$ (18).

Abbreviations: mAb, monoclonal antibody; PS, photosensitizer; SnCe6, Sn(IV) chlorin e6; $^1\Delta_g$, singlet molecular oxygen; Φ_Δ, apparent quantum efficiency of $^1\Delta_g$ generation; Trt, triphenylmethyl protection group; TCPPH, *N*-triphenylmethyl-*N'*-(1-carboxy-5-phthalimidyl pentanoate)hydrazine; HpD, hematoporphyrin derivative; RNO, *N*,*N*-dimethyl-4-nitrosoaniline.

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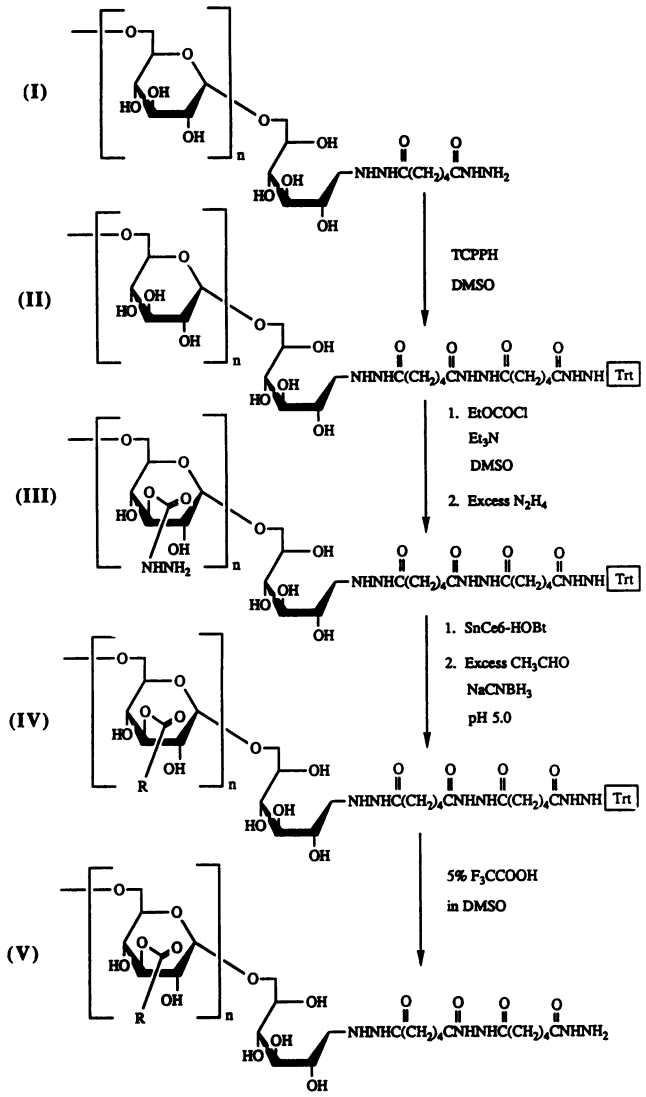

FIG. 1. Diagram of the synthetic scheme used in the preparation of SnCe6-dextran conjugates. Roman numerals identify compounds referenced in the text. Trt, triphenylmethyl protection group; R, NHNH-SnCe6 or $NHN(CH_2CH_3)_2$; HOBt, hydroxybenzotriazole; TCPPH, *N*-triphenylmethyl-*N'*-(1-carboxy-5-phthalimidyl pentanoate)hydrazine; DMSO, dimethyl sulfoxide.

Exhaustive dialysis of the reaction mixture against distilled water followed by lyophilization yielded IV. The Trt protection group in V was removed from the chain-terminal hydrazide group by brief treatment with 5% (vol/vol) trifluoroacetic acid in DMSO. Chromatography of the deprotected SnCe6-dextran on Sephadex G-50 and subsequent lyophilization of the collected void peak produced the SnCe6-dextran module (V). Treatment of V with hot pyridine gave no appreciable loss of SnCe6 bound to the dextran carrier.

mAb Purification. An anti-melanoma mAb 2.1 (a kind gift of R. E. Saxton, University of California at Los Angeles Medical Center) was purified from ascites by affinity chromatography on a protein G column (24). A purified anti-lymphoma mAb, 2.130 (obtained from Damon Biotechnology, Needham, MA), was used as a nonbinding control. Both mAbs were subclass typed as IgG1 antibodies. Antibody purity was checked by HPLC on a DuPont Zorbax GF-250 column eluted with 0.20 M phosphate (pH 7.0) and by SDS/PAGE and isoelectric focusing.

Preparation of Immunoconjugates. Conjugates were prepared by oxidation of the mAb oligosaccharide moiety with 20 mM $NaIO_4$ in 0.15 M acetate buffer (pH 4.75) for 15 min at room temperature (25), followed by addition of ethylene glycol to scavenge excess periodate. The oxidized mAb was passed over a PD-10 (Pharmacia) column equilibrated with acetate buffer to remove low molecular weight by-products and incubated with a 50-fold molar excess of V in acetate buffer at 4°C for 48 hr with the exclusion of light. The conjugates were purified by a two-step procedure using protein G (Pharmacia) affinity chromatography (24) to remove unbound V, followed by chromatography on a Sephacryl S-300 column equilibrated with Dulbecco's phosphate-buffered saline (DPBS) to remove unconjugated mAb. Covalent attachment of the dextran carrier chains to the Fc oligosaccharide of the mAb was assessed using gradient (continuous 8–25% gel) SDS/PAGE in the presence of mercaptoethanol. An apparent molecular weight was estimated by summation of the component molecular weights observed for each reduced immunoconjugate band. In addition, purified conjugates were analyzed by HPLC on a DuPont Zorbax GF-250 column eluted with 0.20 M phosphate (pH 7.0). mAb:PS ratios were determined by γ counting of the ^{113}Sn label in SnCe6 for chlorin content and calculating the corrected absorbance at 280 nm for mAb concentration.

The binding activity of the conjugates relative to the unmodified mAb 2.1 was measured by competitive inhibition RIA using ^{125}I-labeled mAb 2.1 and a suspension of SK-MEL-2 human malignant cells (17).

***In Vitro* Photolysis.** SK-MEL-2 melanoma cells were grown to midlogarithmic phase in 25-cm^2 tissue culture flasks using Dulbecco's modified Eagle's medium (DMEM) (GIBCO) supplemented with 10% fetal bovine serum, glutamine, penicillin, and streptomycin (complete medium). Cells were harvested with 0.05% trypsin and 0.5 mM EDTA, counted, diluted with fresh medium, and plated into triplicate wells in a 24-well plate at a density of 200 cells per well. The cells were allowed to recover overnight in a humidified atmosphere of 5% CO_2 in air at 37°C. Microscopic examination of the wells immediately prior to incubation with the appropriate conjugates showed attached single cells well separated from one another with <10% of the cells found in two or three cell microcolonies.

Medium was removed from each well and replaced with 200 μl of each mAb conjugate solution prepared with a saturating concentration of mAb (100 nM) in complete medium and prechilled to 4°C. The cells were incubated for 45 min at 4°C with the immunoconjugate, washed twice with cold DPBS to remove unbound conjugate, and irradiated at room temperature in DPBS with an argon laser-pumped CW dye laser (Coherent CR-599) at 634 nm (100 mW/cm^2). Light intensity was measured with a calibrated Newport 835 laser power meter. Controls included (*i*) unirradiated cells treated with 2.1 conjugate; (*ii*) irradiated cells exposed to light alone; (*iii*) irradiated and unirradiated cells treated with a nonbinding (anti-lymphoma 2.130) conjugate. Each experiment was performed in triplicate.

Clonogenic assays to determine postirradiation cell survival were performed in the wells where the cells were originally plated (26) in order to avoid artifacts involving postirradiation cell harvesting resistance (27). Cells were washed immediately after irradiation with complete medium and fresh medium was added. The cells were returned to the incubator for 12 days (doubling time of control SK-MEL-2 cells was ≈30 hr), at which point the cells in each well were fixed by addition of glutaraldehyde in DPBS and stained for microscopic observation with 1% toluidine blue. Colonies observed to contain at least 64 cells (six divisions) were scored as survival positive (28).

RESULTS

The spectrum of all conjugates examined (Fig. 2) showed broadening of the Soret and red absorption bands with the

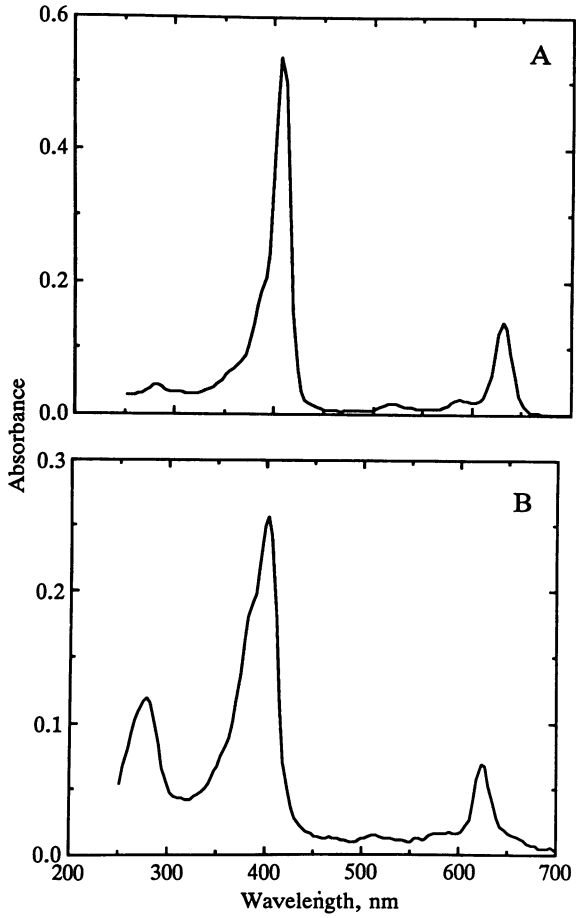

FIG. 2. (*A*) Spectrum of free SnCe6 in DPBS solution (3.05 μM). (*B*) Spectrum of anti-melanoma mAb 2.1-dextran-SnCe6 conjugate (SnCe6:mAb molar ratio, 6.8:1) in DPBS. Note the appearance of a shoulder to the Soret band in the conjugate spectrum.

concurrent appearance of a shoulder at 378 nm in addition to the main Soret absorbance at 409 nm. The blue shift and shouldering of the Soret band are suggestive of hydrophobic interactions between adjacent planar chromophore molecules that have been covalently linked to the dextran carrier. The same spectral behavior was observed for SnCe6-dextran chains alone, suggesting that the random-coiling properties of the dextran carriers allow interaction of bound dyes even though they might be spatially removed from one another. By adjusting the amounts of SnCe6 and dextran, several PS·dextran carriers were constructed that contained from <1 to 15 chromophores per chain (based on average molecular weight for the dextran of 35,000). Because exact quantitation of SnCe6 content by spectrophotometric means was not reliable, a ^{113}Sn-labeled chromophore was prepared. Table 1 provides a list of the conjugates that were prepared along with their constituent SnCe6-dextran and SnCe6-mAb ratios. As shown in Table 1, conjugates with up to 19 chromophores per mAb were prepared.

Characterization of Immunoconjugates. Anti-melanoma 2.1 and anti-lymphoma 2.130 conjugates were prepared and characterized as to their SnCe6 substitution ratios, spectral properties, physicochemical properties, and antigen binding properties. Purified conjugates analyzed by HPLC were found to elute in a single peak corresponding to a molecular weight range of 200,000–240,000. Small amounts of conjugates containing two mAbs per dextran chain were noted in some HPLC analyses. The isoelectric point (pI) of the purified immunoconjugates ranged between 3.8 and 4.0, close to the 3.5 pI of the SnCe6-dextran modules alone.

In order to demonstrate that the SnCe6-dextran chains were covalently attached to the Fc oligosaccharide region of the mAb, SDS/PAGE in the presence of mercaptoethanol was performed. Unmodified light chain bands were found for the conjugated and unconjugated mAbs; however, all of the heavy chain from the purified conjugates migrated to a region of higher molecular weight (≈120,000–140,000) than that seen for unmodified heavy chain (50,000–55,000) (17). Prior to silver staining, the modified heavy chain band was noticeably colored green, indicating comigration of the dye with the modified heavy chain. Since the conjugation chemistry employed would result in a heavy chain protein component of molecular weight 50,000 to which a dextran carrier is coupled (average molecular weight ≈ 35,000), the apparent molecular weight of the modified heavy chain probably cannot be taken as its true molecular weight. Nevertheless, these results indicate that the SnCe6 is bound solely to the heavy chain and are consistent with site-specific linkage of the SnCe6-dextran to the Fc oligosaccharide group of the mAb. A more accurate measurement of the true molecular weight of the conjugate was determined by size-exclusion HPLC of the intact conjugate. A single broad peak eluting in the molecular weight range of 200,000–240,000 was observed.

Conjugate antigen binding properties relative to nonconjugated controls were determined by competitive inhibition RIA using suspensions of SK-MEL-2 human malignant melanoma cells. Nonradiolabeled 2.1 conjugate and unlabeled 2.1 alone (unconjugated mAb) inhibited the binding of unconjugated ^{125}I-labeled 2.1 in a similar manner. All conjugates were found to saturate available cell surface antigens at total mAb concentrations ≥20 nM for a suspension of 250,000 SK-MEL-2 cells (17). The nonbinding conjugate prepared from anti-lymphoma 2.130 exhibited no inhibition of binding of ^{125}I-labeled 2.1 to SK-MEL-2 cell surface antigen.

***In Vitro* Photolysis.** Examination of antibody-targeted photolysis of SK-MEL-2 melanoma cells with two immunoconjugates prepared from anti-melanoma 2.1 and SnCe6-dextran was performed in monolayer culture with SK-MEL-2 human malignant melanoma cells (Fig. 3). Cells were plated from logarithmic phase cultures at low density to allow irradiation

Table 1. Conjugation characteristics of mAbs

mAb	Sample no.	Carrier SnCe6:dextran ratio*	Immunoconjugate SnCe6:mAb ratio†	Dextran chains per antibody	Conjugate yield,‡ %
2.1	1	0.9	1.7	1.89	31
	2	3.5	6.8	1.94	28
	3	5.5	11.2	2.04	27
	4	9.9	18.9	1.91	24
2.130§	1	3.5	7.2	2.25	56

*Determined by using ^{113}SnCe6 to prepare the SnCe6-dextran (molecular weight ≈35,000).
†Determined by using ^{113}SnCe6-dextran and A_{280} (mAb content) corrected for SnCe6-dextran absorbance.
‡Based on the amount of oxidized mAb used in the coupling reaction with ^{113}SnCe6-dextran.
§mAb 2.130 is an anti-lymphoma mAb used as a nonbinding control antibody.

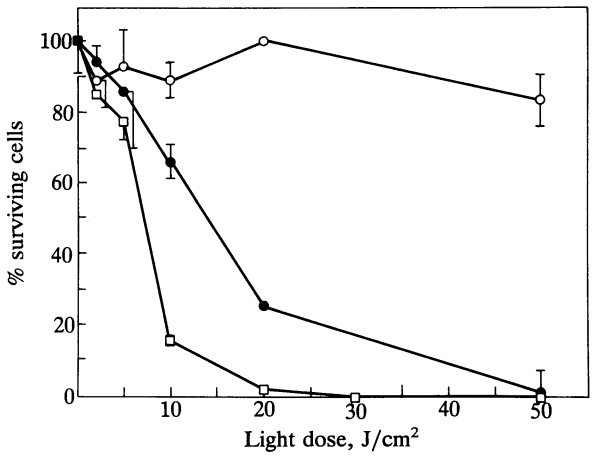

FIG. 3. Survival curves for SK-MEL-2 cells treated with immunoconjugates and light, with cell survival determined by clonogenic assay (mean ± SD, $n = 3$). ●, Anti-melanoma 2.1-dextran-SnCe6 conjugate (SnCe6:mAb molar ratio, 6.8:1); □, anti-melanoma 2.1-dextran-SnCe6 conjugate (molar ratio, 11.2:1); ○, anti-lymphoma 2.130-dextran-SnCe6 conjugate (molar ratio, 7.2:1).

and assay in the same well. A characteristic sublethal region was noted for light doses of <10 J/cm^2 for each conjugate examined. Conjugates completely killed the target melanoma cells at an incident radiation dose of 50 J/cm^2. An identical light dose caused less than a 15% decrease in surviving cell fraction following incubation with a nonbinding conjugate prepared from anti-lymphoma 2.130. Photolysis of the target cells as a function of energy appeared to depend on the SnCe6:mAb molar ratio. The conjugate with the highest ratio (ratio = 11.2) was able to kill half of the cells at a light dose of 8 J/cm^2, whereas an identical level of target cell photolysis required almost 15 J/cm^2 for a conjugate with fewer SnCe6 molecules per mAb (ratio = 6.8).

DISCUSSION

In the present report, SnCe6 was used as the active PS in the selective photolysis of malignant melanoma cells. Several properties of SnCe6 render it a potentially attractive candidate for photodynamic cell killing. First, SnCe6 shows significant long-wavelength absorbance (634 nm), which permits its use in biological systems containing endogenous chromophores that absorb below 600 nm. Second, the apparent quantum efficiency of singlet oxygen generation (Φ_Δ) for SnCe6 has been shown to be 2-fold greater than that of the metal-free chlorin e6 under conditions of matched absorbance at the principal red band for each PS (data not shown). Recent studies of metallo- and metal-free chlorin e6 derivatives have shown that the efficiency of singlet molecular oxygen ($^1\Delta_g$) generation and the chromophore photostability are governed by the identity of the central metal atom (29). Paramagnetic transition metal ions such as Fe(III) and Cu(II) have been found to extinguish $^1\Delta_g$ generation, while the diamagnetic Zn(II) derivative displayed rapid photobleaching. By contrast, the metallo-chlorin e6 derivative used in the present work [containing a diamagnetic Sn(IV) central atom] displayed improved photostability (data not shown) relative to metal-free chlorin e6. Additional advantages in using the metallo-chlorin derivative included (*i*) the option to incorporate a γ-emitting isotope of tin (^{113}Sn) that served as a direct quantifiable label for chlorin content in optically complex biological medium and (*ii*) enhanced water solubility.

Most methods for coupling of active agents of mAbs have employed schemes in which one has little or no control over the site(s) of linkage of the active agent to the mAb (2, 30). In a recent study coupling hematoporphyrin derivative (HpD) to mAb, Steele and coworkers (15) have reported coupling ratios as high as 100 mol of HpD per mol of IgG using a carbodiimide-mediated technique. This ratio is higher than generally reported and is likely to involve chemical (covalent) and physical (adsorptive) mechanisms in the attachment of the PS to the mAb. Techniques that produce significant levels of adsorbed PS may be quite problematic because PS leakage can occur and the amount of PS per mAb may rely on the specific adsorptive properties of the mAb, which may themselves vary considerably.

As a consequence of non-site-specifc linkage methods, reduced activity of the active agent and the mAb has been reported (2, 31, 32). Recently, coupling techniques have been developed that exploit the Fc oligosaccharide residues in mAbs (33). Such methods allow coupling of active agent to the mAb at a point distal to the antigen binding sites, with substantial retention of mAb binding activity and active agent efficacy. However, due to the limited number of oligosaccharide residues available for attachment, the ratio of active agent to mAb using these direct methods is frequently low. For example, using direct attachment of radionuclide chelators to the carbohydrate of mAbs, Rodwell *et al.* (16) reported chelator:mAb ratios of 5.

To increase the ratio of active agent covalently coupled to mAb, polymeric carriers have been used as bridges between the active agent and the mAb (14, 34, 35). We have advanced the previous studies by preparing carriers that contain a single terminal hydrazide allowing the overall conjugation reaction sequence to be better controlled. Although covalent aggregation of the mAb can be prevented by use of a single point of attachment on the carrier polymer, it may still be possible to prevent aggregate formation if a small number of stoichiometrically controlled attachment sites are introduced onto the carrier chain.

Investigation of the antigen binding properties of the conjugate by competitive inhibition RIA showed that the site-specific method used for covalent attachment of the SnCe6-dextran to the mAb preserved substantial antibody binding activity (17). Rodwell *et al.* (16) have shown that mAbs covalently modified by the widely used method of carbodiimide-mediated coupling reactions can result in heterogeneous populations of immunoconjugates exhibiting substantially reduced antigen binding properties, whereas coupling of small molecules to the Fc oligosaccharide moiety yielded reasonably homogeneous and immunoactive conjugates.

Several cell killing experiments were performed with the purified immunoconjugates. Cell killing as a function of increasing light dose (Fig. 3) showed a sublethal damage region below 10 J/cm^2 followed by logarithmic cell killing to nearly complete cell death at higher light doses. The existence of a sublethal damage region for photodynamic cell killing has been established previously for a variety of cells and PS (26, 36). It is difficult to compare our dose–response data with those of others because (*i*) different cell lines, PS, and experimental conditions were used and (*ii*) there are no reliable estimates of how much of the $^1\Delta_g$ generated is actually absorbed by target cells leading to cell damage or death. With respect to the latter point, recent attempts to measure $^1\Delta_g$ *in vitro* using time-resolved spectroscopic techniques (37, 38) may eventually permit direct evaluation of cellular response to light-mediated $^1\Delta_g$ generation.

Although all of the immunoconjugates have proven very efficient in killing target cells, it appeared from other preliminary studies that the bound chromophore (even at chromophore:mAb ratios of unity) was somewhat inferior to the free chromophore in Φ_Δ. For example, in *N,N*-dimethyl-4-nitrosoaniline (RNO) bleaching assays (data not shown), the free chromophore showed an enhanced efficiency of bleaching as compared to that displayed by any of the dextran

or mAb conjugates. Moreover, the spectral changes that occurred when the chromophore was found (Fig. 1) suggest that at least a portion of the bound chromophore may have undergone some substantive changes. These changes might include structural modifications that may have occurred during the immunoconjugate synthesis and/or chromophore aggregation that may occur when chromophores are immobilized on a random coiling macromolecule like dextran. Further characterization of these immunoconjugates is necessary using structural techniques and time-resolved spectroscopy to directly measure the $^1\Delta_g$ for each conjugate as compared to the free SnCe6.

The technique of immunoconjugate preparation described in this report can be used in a wide variety of applications. Because the dextran-PS conjugates are assembled prior to mAb conjugation, a variety of suitably derivatized active agents including other PSs, drugs, and radioisotope chelators can be used without the need for complete modification of the method for each particular agent. Though a practical limit of about 10–15 SnCe6 molecules per dextran chain was obtained using the techniques described in the present work, other less bulky active agents displaying greater aqueous solubility may allow for higher degrees of active agent coupling.

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