ABSTRACT

Photoimmunotherapy is a relatively new and potentially selective experimental approach to the treatment of malignant neoplasms. Its inherent dual selectivity is reinforced by the use of photosensitizer-monoclonal antibody conjugates. The goal of this study was to evaluate the phototoxicity and specificity of an immunoconjugate (IC) synthesized from a chlorin derivative chlorin e₆ monoethylenediamine monoamide (CMA) as the photosensitizer and an anti-ovarian carcinoma monoclonal antibody OC125. Binding efficiency and specificity of the IC were determined by enzyme-linked immunosorbent assay, and specific covalent linkage of the monoclonal antibody to the photosensitizer was demonstrated by fluorescence and electrophoresis. Phototoxicity was tested against ascites or pleural fluid cells from 15 patients with ovarian and nonovarian cancers. Tumor cells from the fluid were treated with the IC at 3 μM equivalent CMA concentration and irradiated at 654 nm (λmax, CMA in IC) at 25 J/cm² from an argon ion-pumped dye laser. Phototoxic efficacy was assayed by [3H]thymidine incorporation. Ovarian cancer cells exhibited high cytotoxicity with [3H]thymidine incorporation of 2.4 ± 2.2%, while nonovarian cancer cells under identical conditions exhibited none to reduced cytotoxicity with [3H]thymidine incorporation of 70 ± 54%. Using a Wilcoxon test, there was a statistically significant difference between these two groups (P < 0.001). Dose-response curves revealed reciprocity in photosensitizer concentration and fluence. These results demonstrate that photoimmunoconjugates retain significant antigen binding specificity and affinity, are effective in the selective photochemical eradication of target cells, and merit further evaluation as photochemotherapeutic agents.

INTRODUCTION

Selective localization of chemicals for diagnostic and therapeutic purposes has been a long sought goal, and has been partially achieved by conjugating small toxic molecules to carrier systems such as proteins, liposomes, and lipoproteins. More recently, improved selectivity has been achieved by a combination of ionizing radiation and ICs. However, systemic toxicity remains a problem because of the poor specificity of all carrier systems known to date. A potentially more selective approach to the treatment of malignant neoplasms is PDT. In PDT, nontoxic photoactivatable compounds, called photosensitizers, are accumulated preferentially in malignant tissue. Exposure to the appropriate wavelength of light causes phototoxicity by the production of active molecular species, such as O₂ (4). The advantage of PDT over conventional therapy is its inherent dual selectivity due to photosensitizer localization in target tissue and spatial control of illuminated areas. Currently Photofrin, a mixture of porphyrins, is the only photosensitizer approved for experimental clinical use in the United States. However, the therapeutic efficacy of Photofrin is limited by a significant skin phototoxicity which persists for 30 to 60 days and low ε in the wavelength region of therapeutic interest (5). Therefore, new photosensitizers (non-Photofrin) with improved specificity and photochemical properties are being investigated.

Improved specificity may also be achieved by the photoimmunotherapy approach (6-12) in which a photosensitizer is linked with tumor specific Mab. However, clinical applications of Mab conjugates are hampered by several considerations. Vascular barriers impair tumor targeting in vivo due to the large size of Mabs and capture by the reticuloendothelial system leading to rapid clearance of these conjugates. In addition, repetitive use of non-human-derived ICs usually elicits an immune response leading to antibodies. These in vivo limitations of ICs can be reduced in extracorporeal treatments such as autologous bone marrow transplantation or in malignancies amenable to intracavitary treatment such as intravesicle or i.p. administration in bladder and ovarian carcinomas, respectively. Our long term goal, the evaluation of the efficacy of photoimmunotherapy in ovarian cancer, takes advantage of the latter in vivo situation.

As a first step toward this goal we have tested the photoimmunotherapy approach on human cancer cells ex vivo. The photosensitizer used is a derivative of chlorin e₆, CMA. CMA is an efficient photosensitizer (quantum yield for O₂ ~ 0.5) with a 10-fold higher ε in the wavelength region of therapeutic interest (λmax, 654 nm) (8, 14) than Photofrin. The Mab used in this study is OC125, which is a murine Mab (15) and recognizes the antigen CA125, expressed in 80% of nonmucinous human ovarian carcinomas (16-19). OC125 is an IgG1 molecule showing positive carbohydrate staining on the heavy chain. In this study we report the synthesis of a covalently bound, carbohydrate site-specific conjugate from CMA and OC125 via PGA functionalized with hydrazine. We also demonstrate that these photoimmunoconjugates retain significant binding specificity and affinity for target cells and are effective in photochemically eradicating ascites carcinoma cells from human ovarian cancer patients while exhibiting reduced phototoxicity to cells from nonovarian cancer patients.

MATERIALS AND METHODS

Monoclonal Antibody and Cells

Fresh tumor cells were obtained from ascites or pleural fluid. The fluid was heparinized and RBC were lysed with 0.83% ammonium chloride (Aldrich Chemical, Milwaukee, WI). Cells were washed with Dulbecco’s PBS (Gibco, Grand Island, NY) and placed in 35-mm Petri dishes at a concentration of 300,000 cells/ml in RPMI 1640 media (Gibco) containing 10% FBS, penicillin, and streptomycin (Sigma, St. Louis, MO). These primary cell cultures proliferated for 2–3 passages.

The OC125 and the NIH:OVCA3 were generous gifts of Centocor (Malvern, PA). The NIH:OVCA3 cell line was derived from the ascites of a patient with ovarian cancer (20). These cells were grown in RPMI 1640 media supplemented with heat-inactivated 10% FBS. The MGH-U1 cell line, a urinary bladder cancer cell line, was a gift of Dr. C. W. Lin, and were cultured and maintained in McCoy’s Medium 5A (Gibco) with 5% heat-inactivated FBS.
Instruments

All irradiations used an argon-ion laser as source (Model Innova 100; Coherent, Palo Alto, CA) pumping a 4-dicyanomethylene-2-

methyl-6-(p-dimethylamino styryl)-4H-pyran dye-containing dye laser (CR-599; Coherent). The 654 nm emission was directed to the cell monolayer as previously described (21).

Spectroscopy. UV-visible spectra were recorded on a diode array spectrophotometer (Model 8451A, Hewlett-Packard, Palo Alto, CA) in quartz cuvets with 1-cm path length.

Gel Electrophoresis. Gel electrophoresis used a Bio-Rad Protein II slab electrophoresis unit. ELISA was conducted on an ELISA reader (Bio-Rad 500 EIA reader).

Synthesis of OC125-PGA-CMA IC

The IC was synthesized by reaction of the carbohydrate moiety at the Mab hinge region as described previously (8, 9, 11–14, 22) away from the antigen-binding sites. The overall synthesis of the IC is outlined in Fig. 1 and described below.

Conjugation of CMA to PGA. The CMA (Porphyrin Products, Logan, UT) was attached to PGA (Sigma) by 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (Sigma) condensation. Typically, PGA (average M, 13,000; Sigma), 5 mg (0.38 µmol) in 2 ml of borate buffer at pH 8.6 was mixed at room temperature with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, 65 mg (0.34 mmol) in 2 ml buffer to activate the PGA carboxy groups. The activated carboxy groups were reacted with 3.5 mg (5.5 µmol) of CMA overnight at 4°C. PGA-bound CMA was functionalized with hydrazine (Sigma) by reaction of the activated ester of PGA (N-hydroxy succinimide ester) with hydrazine hydrate at 4°C for 24 h.

Oxidation of Mab. Typically, 1 mg of Mab (6.67 nmol) in 1 ml of acetate buffer, pH 5.5, was mixed with NaIO4 (Sigma) to a final NaIO4 concentration of 10 mM. The reaction mixture was stirred at 4°C for 0.5 h, at which time 5 ml of 80 mM Na2SO3 (Sigma) were added to quench the reaction. The mixture was allowed to incubate for 5 min at room temperature.

Conjugation of Oxidized Mab to PGA-CMA Conjugate. The functionalized CMA-linked PGA was added to the oxidized Mab solution, such that the molar ratio of CMA:Mab was 50:1, and the mixture was stirred overnight at 4°C in the acetate buffer at pH 5.5. The unreacted carbonyl groups were then quenched with molar excess of ethanolamine (Sigma). The resulting hydrazone was reduced with NaCNBH3 (Sigma). The mixture was allowed to incubate for 5 min at 4°C

Characterization of Immunoconjugate

Mab:CMA ratios were determined from absorbance at 402 nm for CMA concentrations; the IgG was quantitated by protein determination according to the method of Lowry et al. (23).

Covalency and site of linkage of the CMA to OC125 was evaluated by SDS-PAGE essentially according to the method of Laemmli (24). Samples were prepared by dissolving 10–15 µg of Mab in sample extracting solution [0.0125 M Tris-HCl, pH 6.8 (Sigma), 0.4% SDS (Sigma), 2% glycerol (Sigma), 1% 2-mercaptoethanol (Sigma), and 0.0002% pyronin Y]. The gels (10% polyacrylamide SDS running gel and 5% acrylamide stacking gel, Bio-Rad) were run at a constant current and the protein bands were compared to molecular weight standards. The gels were scanned on a CAMAG (Muttenz, Switzerland) densitometer set for absorbance readings at 396 nm for CMA detection and were finally stained with Coomassie blue (Fast Stain; Zinon Research, Alston, MA). CMA fluorescence on the gel was photographed with a long pass filter (cut off < 500 nm). ELISA were performed on the IC and unconjugated OC125. Cells from an established human ovarian cancer cell line (NIH:OVCAR3) and a human bladder cancer cell line (MGH-U1) were used as target and non-target cells, respectively. The assay followed a standard protocol (25) by using horseradish peroxidase conjugated to rabbit anti-mouse F(\mu)2 IgG, IgA, IgM

RESULTS

The absorbance spectra for free CMA, CMA bound to PGA, and the OC125-PGA-CMA IC are shown in Fig. 2. The spectra were essentially similar through the binding steps. In general, PGA-CMA showed a red shift ~5-8 nm compared to free CMA, whereas the IC showed a slight blue shift ~3-5 nm compared to free CMA. The ratio of CMA to Mab was dependent on reactant concentration and was in the 24–40 range under the reaction conditions used. PGA is spectroscopically an ill-behaved polymer with poor compliance to Beer's Law. Quantitations of PGA are, therefore, estimates. The ratio of CMA to PGA was determined by a quantitative enzyme immunoassay (Abbott Laboratories, North Chicago, IL), performed by the chemistry laboratory of the Massachusetts General Hospital.

CA125 Levels. When clinically indicated, serum CA125 levels were determined by a quantitative enzyme immunoassay (Abbott Laborato- ries, North Chicago, IL), performed by the chemistry laboratory of the Massachusetts General Hospital.

<table>
<thead>
<tr>
<th>MAB-(CHOH)</th>
<th>PGA</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaIO4 (10mM)</td>
<td>1. EDC</td>
</tr>
<tr>
<td>30 min. R.T.</td>
<td>2. CMA</td>
</tr>
<tr>
<td>pH 5.5</td>
<td>3. NHS</td>
</tr>
<tr>
<td></td>
<td>4. EDC</td>
</tr>
<tr>
<td></td>
<td>5. H2N-NH2H2O</td>
</tr>
</tbody>
</table>

Fig. 1. Synthesis of OC125-PGA-CMA conjugate. PGA-bound-CMA was functionalized with hydrazine and reacted at the sodium metaperiodate oxidized carbohydrate site on the Mab. EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbo-
diimide; NHS, N-hydroxysuccinimide.

(Zymed, South San Francisco, CA) as the second antibody with an o-
phenylenediamine-H2O2 as substrate.

Phototoxicity

Twenty-four to 48 h after plating, when cell density was approximately 104/ml, cells were incubated with the IC at 3 µM equivalent of CMA in RPMI 1640 medium containing 10% FBS at 37°C in an atmosphere of 5% CO2 for 1 h. Unbound IC was removed by washing 3 times with PBS. Cells were covered with medium containing 10% FBS and irradiated at 654 nm with an argon ion-pumped dye laser with 25 J/cm2. All experiments were performed in duplicate. Twenty-four h following irradiation, phototoxicity to cells was assessed by incorpo-
ration of [H]dThd (New England Nuclear, Wilmington, DE). Cells were pulsed for 16 h with 5 µCi of [H]dThd, washed 3 times with PBS, trypsinized with 0.05% trypsin (Gibco), centrifuged, and washed again with PBS. [H]dThd incorporation was assayed by a liquid scintillation counter (Beckman, Model LS3801). All assays were done in triplicate. Percentage of cell survival was determined from the relation:

\[
\text{Percentage of cell survival} = 100 \times \left(1 - \frac{\text{cpm of treated cells}}{\text{cpm of untreated controls}}\right)
\]
CMA to PGA was estimated from spectroscopic and electrophoretic data to be in the 20-40 range and that of PGA to Mab in the 1-2 range.

Fluorescence analysis of the bound photosensitizer by SDS-PAGE confirmed covalent binding of CMA specifically to the heavy chain of the OC125. Fig. 3 shows the fluorescence of CMA on the heavy chain near an apparent molecular weight of 60,000 in Lane C. Some aggregation of covalently labeled material is apparent at the high molecular weight, possibly representing Mab-PGA-CMA-Mab or CMA-PGA-PGA-CMA cross-linking, Free CMA and PGA-CMA that remained non-specifically bound to the Mab after the Amicon washes migrated at the dye front on the gel.

Results of ELISA with OC125 and OC125-PGA-CMA versus antigen-positive and antigen-negative cells is shown in Fig. 4. The IC, when compared with unconjugated OC125, shows some loss of affinity possibly reflecting the effects of the reaction conditions and polymer folding on the Mab conformation. Both the OC125 and IC showed a much lower degree of binding to the MGH-U1 cells as compared to the target ovarian carcinoma cells, NIH:OVCAR3.

Photoimmunotherapy was first suggested as a potential treatment modality of cancer by Mew et al. (6). Since this initial report, the field has developed gradually but steadily (13); the majority of reports to date have focused on targeting established cell lines. In the present study we demonstrate that this approach may be successfully applied, ex vivo, to human cell systems with a heterogenous cell population.

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**Dose-response curves are shown in Fig. 7. Ovarian cancer cells from patient P. M. were treated with either increasing fluence or with increasing concentrations of IC, resulting in lower $[^3]$HdThd incorporations and higher phototoxicity.

CMA unconjugated to OC125 showed no selectivity for target cells and was equally phototoxic to ovarian and non-ovarian cancer cells (Table 1, Column 4). Mean $[^3]$HdThd incorporation rates were 6.3 ± 10.6 for ovarian cancer cells and 10.1 ± 9 for nonovarian cancer cells.

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Table 1 Percentage of cell survival as determined by [3H]dThd incorporation for the various treatment groups compared with untreated controls

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>Light (%)</th>
<th>IC (%)</th>
<th>IC + light (%)</th>
<th>CMA + light (%)</th>
<th>CA125</th>
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<tbody>
<tr>
<td>M. M.</td>
<td>Ovarian</td>
<td>4.0</td>
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<td>56</td>
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<td>P. M.</td>
<td>Ovarian</td>
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<td>G. R.</td>
<td>Ovarian</td>
<td>0.2</td>
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<td></td>
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<tr>
<td>S. V.</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>M. S.</td>
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<td>6.3</td>
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<td></td>
<td></td>
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<td>N. F.</td>
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<td>A. B.</td>
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<tr>
<td>H. N.</td>
<td>Borderline</td>
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<tr>
<td>J. M.</td>
<td>Unknown primary</td>
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<tr>
<td>A. V.</td>
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<td>8.8</td>
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<tr>
<td>G. O.</td>
<td>Gastric</td>
<td>100.0</td>
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<tr>
<td>D. P.</td>
<td>Lung</td>
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<tr>
<td>E. L.</td>
<td>Breast</td>
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<td>7.3</td>
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<tr>
<td>E. G.</td>
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<td>E. C.</td>
<td>Colon</td>
<td>73.0</td>
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</table>

* Phototoxicity was tested against ascites or pleural fluid cells from 15 patients with ovarian and nonovarian cancers. Tumor cells were treated with IC or free CMA at 3 μM equivalent CMA concentration and irradiated at 654 nm (λmaxCMA) at 25 J/cm² from an argon ion-pumped dye laser. Phototoxic efficacy was assayed by [3H]dThd incorporation, and compared to untreated controls; cpm for controls ranged from 1 to 4 x 10^5. Statistical analysis was performed by using a Wilcoxon test.

† Treatment with light alone.
‡ Treatment with IC alone in the absence of light.
§ No cytotoxicity with CMA without light.
¶ Serum CA125 levels in units/ml; normal levels < 35 units/ml.
# End stage ovarian cancer resistant to chemotherapy.

Phototoxicity was assessed by decreased [3H]dThd incorporation of cells treated with OC125-PGA-CMA IC and 654 nm irradiation. Columns, percentage of untreated controls; bars, SD. Statistical analysis was performed by using a Wilcoxon test.

Fig. 5. Phototoxicity of ovarian and nonovarian cancer cells. Phototoxicity was assessed by decreased [3H]dThd incorporation of cells treated with OC125-PGA-CMA IC and 654 nm irradiation. Columns, percentage of untreated controls; bars, SD. Statistical analysis was performed by using a Wilcoxon test.

Fig. 6. Phototoxicity plotted against CA125 levels. Normal CA125 level less than 35 units/ml. Phototoxicity assessed by decreased [3H]dThd incorporation. Ca, cancer.

CMA, although immunoreactivity of Mab is reduced. The shifts in the absorption spectra of the conjugated CMA relative to the unconjugated molecule represent changes in the photophysics consequent to binding and are not surprising. The binding of photosensitizer to macromolecules may increase the triplet population due to a decrease in the singlet-triplet splitting (26). The red shift observed with PGA-CMA is consistent with such an alteration in energy levels. The blue shift observed with the IC on the other hand, may be attributed to aggregation of the CMA molecules in the sterically more complex and restricted environment of the Mab. Consistent with this observation is the report demonstrating aggregation of certain carbocyanine dyes in the presence of added protein (27). The SDS-PAGE gives only apparent molecular weights and the appearance of the fluorescent band at approximately M, 60,000 rather than the expected M, 70,000–80,000 is probably due to the significant modifications in the physical behavior of the Mab that must occur as a consequence of conjugation with the hydrophobic CMA molecules and the negatively charged PGA molecules.

Phototoxicity against cells from ovarian and nonovarian cancer patients showed that the IC was selectively phototoxic to ovarian cancer cells. Ascites cells represent a mixed population with non-uniform antigen expression. Therefore, the high levels of cytotoxicity observed in this study was surprising and could

Fig. 7. Dose-response curves for fluence and PS concentration dependence. ●, Patient P. M. cells treated with OC125-PGA-CMA IC (3 μM in CMA) and exposed to increasing fluence (J/cm²). □, Patient P. M. cells treated with increasing amounts of OC125-PGA-CMA IC and exposed to a constant fluence of 25 J/cm². Phototoxicity was assessed by decreased [3H]dThd incorporation. Points, percentage of untreated controls; bars, SD.
PHOTOIMMUNOTHERAPY AND OVARIAN CANCER

arise from the “near neighbor effect” in which active molecular species produced on the cell surface of antigen-positive cells may cause death of antigen-negative cells by diffusion. The phototoxicity of the IC to nonovarian cells is probably due to nonspecific interactions of the IC with non-target cells, the expression of CA125 on certain nonovarian cancers, and “impurities” (PGA-CMA that remains noncovalently bound after the Amicon washes).

Free CMA, which is not preferentially retained by malignant tissue, showed no selectivity in its phototoxicity, as expected. [3H]dThd incorporation ranged from 1 to 30% compared with untreated controls for both ovarian and nonovarian cancer cells. These data demonstrate that site-specific conjugation of the CMA to OC125 allows it to be selectively cytotoxic and that the conjugation process does not affect the ability of CMA to act as a photosensitizer.

OC125 recognizes a Mr 200,000 immunoreactive glycoprotein, the cell surface antigen CA125 (16–19). Serum levels of CA125 are currently used in the diagnosis of ovarian cancer. While CA125 levels are elevated with ovarian cancer (15–17), the antigen is not unique to this disease. CA125 is found in human milk (18) and is associated with other malignancies (19, 28), surgery, and pregnancy (19, 29). In this study, CA125 levels showed qualitatively an essentially linear relationship with phototoxicity in the case of nonovarian cancer cells. Because it was clinically unindicated, serum CA125 levels were not available for patients with breast cancer; however, reports in the literature indicate that between 15 and 30% of patients with breast carcinoma have elevated serum CA125 levels (19, 28, 29). It is conceivable that the relatively high phototoxicity seen with cells from the two breast cancer patients is due to an elevated expression of CA125. The two patients in this study with 100% [3H]dThd incorporation both had lung cancer, one squamous cell and one adenocarcinoma, with a CA125 level of 8.8 units/ml in the latter. The two nonovarian cancer patients with the lowest [3H]dThd incorporation and the most significant cytotoxicity (J. M., E. C.; Table 1) both had CA125 levels of 7–8 times normal. This direct correlation between cytotoxicity and serum CA125 levels for the nonovarian cancer cells again demonstrates photochemical destruction with antigen selectivity. Cells from ovarian cancer patients exhibited high phototoxicity regardless of the serum CA125 level, reflecting the high level of antigen expression on the cell surface.

Cancer patients who develop resistance to one treatment modality (i.e., chemotherapy) frequently will have no response to a second modality (i.e., radiation therapy) (30, 31). Although the molecular nature of this cross-resistance remains unclear, the mechanisms are probably multifactorial, involving alteration in drug accumulation, DNA repair abilities, and glutathione levels (30). A significance of this study is that cells from patients with chemoresistance showed excellent response to PDT, suggesting a different mechanism of action and the potential utility of PDT for cases where resistance to chemotherapy has developed.

Photoimmunoconjugates have in recent years been shown to be effective (6–14) in the photochemical destruction of established cell lines which represent a homogenous population of antigen-positive cells. In a recent study (32), we examined the phototoxic efficacy of the IC against an established human ovarian carcinoma cell line, NIH:OVCAR3 (target cells) and a human keratinocyte cell line (nontarget cells). The ovarian cancer cells showed only a 2 ± 2% [3H]dThd incorporation compared with untreated controls, while under identical conditions, keratinocytes showed a 65 ± 5% [3H]dThd incorporation. Our present investigation is the first study testing photoimmunotherapy in a heterogeneous cell population directly from patients. The results from this investigation suggest that photoactivation of ICs has potential for treatment of ovarian cancer, which is the fifth most frequently occurring fatal cancer in the United States. Because in the majority of patients with ovarian cancer the disease is confined to the peritoneal cavity, it is well suited for photoimmunotherapy. The peritoneal surfaces can easily be exposed to laser irradiation via optical fibers during laparotomy or laparoscopy. Additionally, i.p. injection of ICs would be expected to reduce the problem of rapid reticuloendothelial system clearance. Applications of non-immuno-PDT to the experimental treatment of ovarian cancer with hematoporphyrin derivative have been reported. Tochner et al. (33, 34) using a murine i.p. ovarian ascites tumor model produced cures in 85% of the animals. These results have lead to Phase I human clinical trials with hematoporphyrin derivative-mediated PDT (35), although problems with light delivery and dosimetry remain to be sorted out.

In summary, our experiments demonstrate that photochemical destruction with antigen selectivity can be achieved with a Mab-photosensitizer conjugate. In addition, cells which are resistant to conventional chemotherapy are sensitive to photodynamic therapy. Our data suggest that the approach merits further investigation. Progress in this field will be made by improving the synthesates, purification, and characterization of ICs and by optimizing light dosimetry and delivery in appropriate animal models.

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Photoimmunotherapy of Human Ovarian Carcinoma Cells ex Vivo

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