Synergistic Interaction between Anti-p185\textsuperscript{HER-2} Ricin A Chain Immunotoxins and Radionuclide Conjugates for Inhibiting Growth of Ovarian and Breast Cancer Cells That Overexpress HER-2\textsuperscript{1}

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ABSTRACT

Radionuclide conjugates or ricin A chain (RTA) immunotoxins that target p85\textsuperscript{HER-2} have partially inhibited the growth of human ovarian cancer xenografts in athymic mice but generally have not cured mice bearing human tumor transplants. The present study was undertaken to explore whether a combination of ionizing radiation and an immunotoxin could exert additive or synergistic cytotoxicity in culture and in vivo against cancer cells that overexpress p85\textsuperscript{HER-2}. In cell culture, treatment with 200–2000 cGy external beam irradiation followed by incubation with TA1-anti-p85\textsuperscript{HER-2}-RTA immunotoxin (TA1-RTA) produced synergistic inhibition of clonogenic growth of ovarian and breast cancer cells that expressed >10\textsuperscript{6} p85\textsuperscript{HER-2} receptors/cell. The effect on cell survival correlated with an inhibition of DNA repair. A prior study (F. J. Xu et al., Nucl. Med. Biol., 24: 451–460, 1997) compared the biodistribution of radionuclide conjugates prepared with monoclonal antibodies that bind to different epitopes on the extracellular domain of p85\textsuperscript{HER-2} and found optimal tumor uptake with the 520C9 antibody, which did not compete with TA1 for binding to the receptor. In this report, the TA1-RTA immunotoxin and the 131I-labeled 520C9 radionuclide conjugate could each inhibit the growth of clone-9002-18 xenografts in athymic mice but did not yield long-term survivors using maximally tolerated doses of each agent. When TA1-RTA and 131I-labeled 520C9 were used in combination, a greater inhibition of tumor growth was obtained than with either single agent. Similarly, survival with the combined treatment was significantly prolonged ($P = 0.004$) relative to treatment with immunotoxin or radionuclide conjugate alone. After treatment with an optimal combination of immunotoxin and radionuclide conjugate, 50% of mice survived >300 days, whereas controls succumbed with a median survival of 36 days. These results suggest that combinations of immunotoxins and radionuclide conjugates deserve further evaluation for the treatment of cancers that overexpress p85\textsuperscript{HER-2}.

INTRODUCTION

Over the last two decades, monoclonal antibodies and their conjugates have begun to contribute to the management of several forms of human cancer. Treatment with unconjugated antibodies has produced objective regression in a fraction of leukemias (1), lymphomas (2, 3) and breast carcinomas (4). An anti-p185\textsuperscript{c-erbB-2} antibody has potentiated the activity of cytotoxic drugs in patients with recurrent breast cancer (5). The therapeutic potency of antibodies has been increased by conjugation with radionuclides. Using radioimmuno-therapy, durable responses have been achieved in lymphomas that had proven refractory to conventional agents (6, 7). Phase II clinical trials suggest that yttrium-90-labeled anti-human milk-fat-globule protein (HMFG1) antibody can produce objective responses in patients with epithelial ovarian cancer (8). At present, the impact on survival of the i.p. administration of yttrium-labeled anti-human-milk-fat-globule protein antibody is being evaluated in a multinational Phase III trial in patients who have had a complete clinical response to conventional therapy.

To improve the clinical efficacy of radioimmuno-therapy, radionuclide conjugates might be used in combination with immunotoxins that recognize different antigens or epitopes expressed on the same cancer cells. Immunotoxins have potentiated the cytotoxic activity of alkylating agents (9) and might also potentiate radiation damage. Synergy has also been observed between paclitaxel and radioimmuno-therapy with yttrium-90-labeled chimeric antibody (10). Among the antigenic targets associated with breast and ovarian cancers, p85\textsuperscript{HER-2} is overexpressed in up to 30% of cases, and this can be associated with a poor prognosis (11, 12). Availability of multiple antibodies that react with antigenically distinct epitopes on the extracellular domain of p85\textsuperscript{HER-2} has permitted the targeting of a single receptor by multiple therapeutic approaches including antibody alone, radionuclide conjugates, and immunotoxins.
Unconjugated antibodies against some, but not all, epitopes on the extracellular domain of pl85HER-2 can inhibit clonogenic growth of cells that overexpress the receptor (13). At optimal concentrations of unconjugated antibody, however, only 90% inhibition of clonogenic growth can be achieved. Treatment with anti-pl85HER-2 antibodies that have been conjugated with RTA can inhibit growth by 99.99%, i.e., a reduction by some 4 logs of clonogenic tumor cells (14). Optimal cytotoxicity of anti-pl85HER-2-RTA conjugates depends critically on the density of pl85HER-2 receptors on each tumor cell. Tumor cells with >10^6 copies of pl85HER-2 were most markedly inhibited, whereas tumor cells with 10^5 copies exhibited less than one log of inhibition (14). Because normal nonmalignant tissues exhibit, at most, 10^4 copies of pl85HER-2 per cell, a therapeutic window might exist for treatment with immunotoxins in vivo.

In vitro and in vivo models have been established to test the activity of immunotoxins and radionuclide conjugates that target cells with a high density of pl85HER-2. SKOV3 ovarian cancer cells have been transfected with additional copies of HER-2, and clones have been isolated that express >10^6 copies of pl85HER-2. Clone-9002-18 cells overexpress pl85HER-2 and have retained the ability to grow both in cell culture and as xenografts in athymic nu/nu mice. Using clone-9002-18 we have asked: (a) whether a combination of external beam radiation and immunotoxin might have additive or synergistic activity against cells that overexpress pl85HER-2; (b) what mechanism(s) might contribute to this interaction; and (c) whether similar additive or synergistic interactions might occur in vivo between anti-pl85HER-2-RTA3 immunotoxin and anti-pl85HER-2 radionuclide conjugates.

MATERIALS AND METHODS

Monoclonal Antibodies. Murine monoclonal antibodies TA1 and 520C9 that react with the extracellular domain of pl85HER-2 were obtained, respectively, from Applied BioTechnology/Oncogene Science (Cambridge, MA; Ref. 15) and from Chiron, Inc. (Emeryville, CA; Refs. 16, 17). All of the antibodies were of the IgG1 isotype, MOPC21 (IgG1), an isotype-matched control, did not bind to pl85HER-2. To prepare antibodies, hybridoma cells were washed free from serum and injected i.p. into pristane-primed BALB/c mice. When teneascites had formed, fluid was harvested aseptically. IgG antibodies were purified from hybridoma-induced ascites fluid using protein A-Sepharose chromatography (Pharmacia LKB, Uppsala, Sweden). Fractions containing protein measured by absorbance at 280 nm were dialyzed for 24 h against 50 mM phosphate buffer (pH 7.4) and were concentrated using an Amicon filter and compressed nitrogen gas. Immunoglogulin concentration was calculated by dividing absorbance at 280 nm by the extinction coefficient for IgG. Immunoglobulin purity was confirmed by SDS PAGE. Purified immunoglobulin was aliquoted and stored at −70°C.

Radioiodination of Monoclonal Antibodies. Monoclonal antibodies were labeled with Na131I using the iodogen method (18). In brief, 50 μl of phosphate buffer [0.5 M (pH 7.4)] was added to a 15 × 75-mm borosilicate tube coated with 10–100 μg of iodogen (Pierce Chemical Co. Rockford, IL). Monoclonal antibody (50–500 μg) was added in a volume of 95 μl of PBS [50 mM phosphate buffer and 0.15 M NaCl (pH 7.4)]. Radioiodination was initiated by the addition of 0.5–10 mCi of Na131I, and the mixtures were incubated for 30 min on ice. The protein-bound iodine was separated from free 131I by gel filtration on a PD-10 column (Pharmacia, Pleasant Hill, CA) equilibrated with PBS. A sample of 3 μl from each fraction was counted in a Packard gamma counter (Packard Instrument Company, Downers Grove, IL) to measure protein-bound radioactivity. Iodination efficiency ranged between 75 and 90%, and the specific activity was >6 μCi/μg.

Preparation of Immunotoxin. The TA1 and 520C9 murine monoclonal antibodies were conjugated with RTA using 2-iminothiolane as described previously (19).

Cell Line. SKOV3 9002-18 (clone-9002-18) ovarian cancer cells and SKBr3 breast cancer cells were maintained in TCM, consisting of McCoy 5A medium supplemented with 10% FBS, 2 mM l-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 400 μg/ml G418 (Life Technologies, Inc., Grand Island, NY). Clone-9002-18 cells were produced by transfection of the full-length human HER2/neu (c-erbB-2) gene into the SKOV3 cell line, to provide a subline that expressed 2 × 10^6 pl85HER-2 binding sites per cell (14).

Serial Dilution Clonogenic Assay and Isobolographic Analysis. Cytotoxicity was evaluated using a limiting dilution technique as described previously (20). After trypsinization, 10^6 tumor cells were irradiated and/or incubated with immunotoxin for 3 h in a total volume of 1 ml. A Csium-137 irradiator was used for external beam irradiation delivering 27 cGy/min for total doses <200 cGy and 3904 cGy/min for doses ≥200 cGy. Cells were then washed twice with TCM. A series of nine 5-fold dilutions were prepared. Six aliquots (100 μl) of each dilution were plated in 96-well flat-bottomed microtiter plates preloaded with 100 μl of TCM. Plates were incubated for 14 days at 37°C, in 5% CO2 and 95% humidified air. Growth of colonies (>50 cells) was evaluated by visual scoring. Each value was calculated from a mean of duplicate plates. Limiting-dilution analysis was then performed (20).

Isobolographic analysis, a geometric method to explore drug interactions, was performed as described by Berenbaum (21) and Steel and Peckham (22). Isoboles for different levels of cytotoxicity were drawn from dose-response curves, in which the log effect by dose of one agent was plotted for each constant dose of the other agents in the combination. The calculation of an “envelope of additivity” between modes I and II, which indicated the theoretical limits of the additive effects obtained from an interaction of two agents. An interaction was considered to be synergistic when the combined cytotoxic effects exerted by two different agents fell below the envelope. In the case of the MOPC-RTA control, traditional isobolographic analysis could not be used to compare the combined effects of radiation and MOPC-RTA because the effect of increasing doses of MOPC-RTA on cell survival was not monotonely decreasing. Consequently we asked whether there was evidence of decreased (or increased) clonogenic growth with increasing concentrations of the immunotoxin at different levels of irradiation.

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3 The abbreviations used are: RTA, ricin A chain; TCM, tissue culture medium; BrdUrd, 5-bromodeoxyuridine; FdUrd, fluorodeoxyuridine.
Differences in log surviving fraction between that found at zero concentration of immunotoxin and at levels of 0.1, 0.25, 0.5, and 1.0 μg/ml of MOPC-RTA were fitted by linear regression (23) for each fixed level of radiation. Thus the differences in log cell survival were fitted as:

\[ \log SF(R,0) - \log SF(R,D) = a + bD \]

where \( SF(R,D) \) is the surviving fraction at the radiation level \( R \) with MOPC-RTA at dose \( D \) and where \( a \) and \( b \) are constants. For each radiation dose, the slope \( b \) and the uncertainty in the estimate of \( b \) were calculated and compared.

**Measurement of Repair Synthesis.** DNA of cultured cells was prelabeled by growing cells for 7 days in medium containing 0.01 μCi/ml \(^{14}\text{C}\)thymidine. For repair analysis, \(^{14}\text{C}\)thymidine-prelabeled cultures were incubated for 1 h before treatment in 10 μM BrdUrd and 1 μM FdUrd, washed with PBS, and irradiated or sham-irradiated with 600 or 2000 cGy of γ irradiation at a dose rate of 1 Gy/min. After irradiation, the cells were allowed to repair in medium containing 10 μM BrdUrd, 1 μM FdUrd, and 30 μCi/ml \(^{3}\text{H}\)thymidine (82 Ci/mmol) in the presence or absence of the immunotoxin. After 4 h, the medium was removed from the plates, the cultures were washed twice with PBS, and the cells were lysed in 10 mM Tris-HCl (pH 8.0) and 10 mM EDTA with 0.5% SDS.

Repair synthesis was measured as described by Smith *et al.* (24) by first resolving parental density DNA (containing \(^{3}\text{H}\)thymidine and BrdUrd substituted repair patches) from hybrid density DNA (synthesized by semiconservative replication) by centrifugation in CsCl gradients. The parental density DNA was further purified in a second neutral CsCl gradient. \(^{14}\text{C}\) activity was assayed and the DNA concentration determined spectrophotometrically by measuring the absorbance at 260 nm. \(^{14}\text{C}\) specific activity was calculated from the same material, permitting conversion of \(^{14}\text{C}\) activity in double-labeled samples to μg DNA before plotting the ratio of \(^{3}\text{H}\)-labeled cpm/μg of DNA. Repair synthesis was calculated from the isolated parental density DNA as the ratio of \(^{3}\text{H}\)-thymidine cpm per μg of DNA.

**Growth Inhibition of Clone-9002-18 in nu/nu Mouse Xenografts.** Aliquots of \(2 \times 10^7\) clone-9002-18 cells that had been grown in tissue culture were trypsinized, washed, and injected s.c. into 20-g athymic BALB/c nu/nu mice (Charles River) in a volume of 0.1 ml. Palpable tumors generally formed 4–5 days after injection. Animals were divided into groups of 5–6 mice. On days 5, 6, and 7 after the injection of tumor cells, TA1-RTA immunotoxin (50 or 75 μg/day) was injected i.p. in a volume of 0.5 ml HBSS into some groups. On day 5 after injection of tumor cells, \(^{131}\text{I}\)labeled antibody was estimated from a previous study (25) in which the tissue distribution of this labeled antibody was determined as a function of time. From these data, the tumor-absorbed dose was calculated using standard MIRD formulation. Control groups received injections of HBSS by the same routes and according to the same schedules. Tumor size was measured as the product of two perpendicular diameters every 3 days. Mice were killed when tumor size reached 2 cm or when they appeared to be in distress.

**Statistical Analysis.** The significance of differences in tumor size was determined with the Student *t* test. Differences in survival were evaluated with the Wilcoxon Mann-Whitney rank order test.

**RESULTS**

**Synergistic Interaction between Anti-p185HER-2 Immunotoxin and Ionizing Radiation in Cell Culture.** In previous studies, the potency of several immunotoxins had been compared after conjugating RTA with murine monoclonal antibodies against different epitopes on the extracellular domain of
p185\textsuperscript{HER-2}. Optimal inhibition of clonogenic growth was observed with TA1-RTA (26). Cytotoxicity depended on the concentration of immunotoxin (26) and on the density of p185\textsuperscript{HER-2} expression (14). When clone-9002-18 ovarian cancer cells that expressed $10^6$ p185\textsuperscript{HER-2} receptors/cell were tested, an optimal concentration of TA1-RTA immunotoxin (5 \mu g/ml) could inhibit growth of clonogenic tumor cells by 3.8 logs (Fig. 1A).

Treatment with a mixture of unconjugated TA1 (4.2 \mu g/ml) and free RTA (0.8 \mu g/ml) inhibited only 0.1 log of clonogenic growth in an experiment in which TA1-RTA (5 \mu g/ml) reduced clonogenic cells by 3.8 logs (data not shown).

To evaluate interactions of ionizing radiation and immunotoxin, we have treated clone-9002-18 cells with different doses of \gamma radiation before incubation for 3 h with different concentrations of TA1-RTA. As would be anticipated with mammalian cells, inhibition of clonogenic growth was observed in a log-linear dose-dependent manner after treatment with 20–2000 cGy (Fig. 1B). When clone-9002-18 cells were treated with a combination of immunotoxin and \gamma radiation, increasing concentrations of TA1-RTA immunotoxin (0.1–1.0 \mu g/ml) produced progressively greater cytotoxicity in combination with \gamma irradiation (Fig. 1C). Isobolographic analysis indicated that the two modalities interacted synergistically with an isobole that fell below the envelope of additivity (Fig. 2).

Similar synergy was observed between \gamma radiation and the 520C9-RTA immunotoxin that recognized a different epitope on the extracellular domain of p185\textsuperscript{HER-2} (data not shown). Unconjugated TA1 antibody (Fig. 1C), 520C9 antibody (data not shown) or nonspecific MOPC antibody (data not shown) failed to potentiate the response to \gamma irradiation. Similarly, MOPC-RTA did not interact with radiation. In two replicate experiments (data not shown), MOPC-RTA killed less than 0.5 logs of clonogenic cancer cells over a wide range of concentrations (0.1–1.0 \mu g/ml). Linear regression analysis was performed for different MOPC-RTA concentrations (0.1–1.0 \mu g/ml) at each of several levels of radiation (0–2000 cGy). In every case, the slope $b$ was less than the uncertainty of the slope, indicating that there was not enough information to show that there was any interaction between MOPC-RTA and radiation. Interestingly, a 3-h treatment with the protein synthesis inhibitor cyclohexamide (10^{-2}–10^{-7} M) failed to potentiate the effect of radiation on clonogenic growth (data not shown).

To determine whether synergy would be observed between ionizing radiation and immunotoxin against other cell lines that overexpressed p185\textsuperscript{HER-2}, experiments were performed with the SKBr3 breast cancer cell line that expressed $10^6$ copies of the receptor per cell. Synergistic interactions were documented in three of four experiments with clone 18 ovarian cancer cells and in three of seven experiments with SKBr3 breast cancer cells (data not shown). Difficulty in demonstrating a consistently synergistic interaction between radiation and immunotoxin with SKBr3 cells seemed related to the fact that the SKBr3 cell line was more sensitive to radiation than was clone-9002-18. After treatment with 600 cGy in
synergy experiments, 0.84 logs of clone-9002-18 were eliminated compared with 2.19 logs of SKBr3 (P = 0.003; Wilcoxon Mann-Whitney rank order test). When lower doses (20–1000 cGy) of radiation were evaluated, synergy was observed in two of three experiments with SKBr3 cells.

**Immunotoxin-mediated Inhibition of Radiation-induced DNA Repair.** DNA repair was measured by the incorporation of [3H]thymidine after irradiation (600–2000 cGy). Repair replication was induced by irradiation in a dose-dependent manner (Fig. 3). Subsequent incubation with TA1-RTA (0.1–0.5 μg/ml) for 4 h inhibited radiation-induced repair. Higher doses of TA1-RTA produced greater inhibition of radiation-induced DNA repair. Similar results were obtained with clone-9002-18 ovarian cancer cells and with SKBr3 breast cancer cells that also expressed >10^6 p185HER-2 receptors per cell.

**Synergistic Antitumor Activity in Vivo Using a Combination of p185HER-2 Immunotoxin and Radionuclide Conjugate.** When RTA immunotoxins were prepared with different anti-p185HER-2 antibodies, optimal inhibition of clone-9002-18 growth in cell culture had been obtained in previous studies with TA1-RTA immunotoxin (26). To evaluate the impact of TA1-RTA treatment in vivo, clone-9002-18 ovarian cancer cells were grown as xenografts in athymic BALB/c nu/nu mice. Transplants of 2 × 10^7 clone-9002-18 cells grew regularly after s.c. injection and formed palpable tumors within 5 days. In a dose-finding study, groups of five mice with s.c. tumor transplants were treated i.p. with TA1-RTA at 50 or 75 μg per day on days 5, 6, and 7 after transplant (data not shown). At the higher dose, one of five mice died of acute toxicity 11 days after transplant and 4 days after the last injection of immunotoxin. In subsequent studies, 50 μg/day on each of 3 consecutive days was considered a maximally tolerated dose of TA1-RTA immunotoxin. TA1-RTA significantly inhibited s.c. tumor growth (P < 0.05) in both groups during days 11 and 28, but complete regression of xenografts was not attained (Fig. 4). Similar antitumor activity was observed with the two dose levels of TA1-RTA.

Because synergistic interactions had been observed between radiation and immunotoxin in cell culture, we evaluated the antitumor activity of TA1-RTA immunotoxin, alone and in combination with a different anti-p185HER-2 antibody that had been labeled with ^131I. In earlier studies, radiolabeled 520C9 exhibited optimal tumor targeting of clone-9002-18 xenografts in athymic nu/nu mice (25). Because TA1 and 520C9 recognized distinct epitopes on the extracellular domain of p185HER-2 (13), the two conjugates could be used in combination to evaluate the possibility that additive or synergistic interactions between radiation and immunotoxin might be observed in vivo as well as in cell culture.

Groups of six mice with established tumor transplants were treated with: (a) diluent; (b) 50 μg/day TA1-RTA (days 5, 6, and 7); (c) ^131I-labeled 520C9 (100 μCi or 250 μCi on day 5); or (d) both immunotoxin and radionuclide conjugate. For injected activities of 100 and 250 μCi, it is estimated that these xenografts received 766 and 1916 rads, respectively. The ^131I-labeled 520C9 inhibited tumor growth at a maximally tolerated dose of 250 μCi/mouse (Table 1) but failed to produce long-term survival in two replicate experiments (Table 2). When TA1-RTA (50 μg/mouse on days 5, 6, and 7) was combined with ^131I-labeled 520C9 (100 or 250 μCi/mouse on day five), significant growth inhibition was attained (P < 0.001). The higher delivered dose of radionuclide (1916 rads) seemed more effective than the lower dose (766 rads) when used as a single agent or in combination with TA1-RTA (Fig. 5). In the first experiment, all of the mice that were tumor-free at 34 days were followed long-term, and in the second experiment all of the animals were followed for progressive tumor growth. Survival was modestly but significantly prolonged with each of the individual agents (Fig. 6). When immunotoxin and radionuclide conjugate (100 μCi or 250 μCi) were combined, survival was significantly better (P = 0.004) than that attained with either single agent. The optimal combination of immunotoxin (50 μg TA1-RTA every day for 3 days) and radionuclide conjugate (250 μCi ^131I-labeled 520C9) extended median survival from 36

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**Table 1** Growth inhibition of s.c. clone-9002-18 ovarian cancer xenografts after treatment with TA1-RTA immunotoxin and ^131I-labeled 520C9 radionuclide conjugates, alone and in combination

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Control</th>
<th>TA1-RTA</th>
<th>^131I-labeled 520C9 (100 μCi)</th>
<th>TA1-RTA + ^131I-labeled 520C9 (100 μCi)</th>
<th>TA1-RTA + ^131I-labeled 520C9 (250 μCi)</th>
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<tbody>
<tr>
<td>1st</td>
<td>1.01 ± 0.08</td>
<td>0.73 ± 0.10</td>
<td>0.87 ± 0.20</td>
<td>0.64 ± 0.29</td>
<td>0.48 ± 0.38</td>
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<tr>
<td>2nd</td>
<td>1.26 ± 0.08</td>
<td>0.71 ± 0.16</td>
<td>0.69 ± 0.04</td>
<td>0.48 ± 0.13</td>
<td>0.27 ± 0.21</td>
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</table>

*Groups of six mice received 10^7 clone-9002-18 cells s.c. and were treated with TA1-RTA (50 μg) i.p. on days 5, 6, and 7 and/or with ^131I-labeled 520C9 (100 μCi or 250 μCi) i.v. on day 5. Controls received injections of HBSS at the same intervals.

*Measurements were made on day 30–31 after transplantation of clone-9002-18. Data are expressed as mean tumor diameter (cm) ± SD.

*Combination differs from individual agents (P < 0.01).
Table 2  Long-term survival of nu/nu mice with clone-9002-18 ovarian cancer xenografts after treatment with TA1 RTA immunotoxin and 131I-labeled 520C9 radionuclide conjugate, alone and in combination

Mice were treated as described in Table 1. In experiment 1, mice with measurable tumors were killed on day 34, but mice that had achieved complete regression of tumor transplants were observed up to 300 days. In experiment 2, all of the mice were observed up to 300 days.

<table>
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<th></th>
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<td>6/6</td>
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<tr>
<td>(100 μCi)</td>
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<td>6/6</td>
<td>9/12</td>
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<tr>
<td>(250 μCi)</td>
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* Exp, experiment.

Fig. 5 Effect of TA1-RTA and 131I-labeled 520C9, individually and in combination, on the growth of clone-9002-18 xenografts in nu/nu mice. Groups of six mice were treated with diluent, TA1-RTA (50 μg/mouse on days 5, 6, and 7), 131I-labeled 520C9 (100 μCi/mouse on day 5), 131I-labeled 520C9 (250 μCi/mouse on day 5), TA1-RTA (50 μg/mouse on days 5, 6, and 7) plus 131I-labeled 520C9 (100 μCi/mouse on day 5), or TA1-RTA (50 μg/mouse on days 5, 6, and 7) plus 131I-labeled 520C9 (100 μCi or 250 μCi/mouse on day 5). Relative to diluent controls, tumor growth was significantly inhibited (P < 0.01) by TA1-RTA, 131I-labeled 520C9 (250 μCi), and the combination of TA1-RTA and 131I-labeled 520C9 (100 μCi or 250 μCi) on days 10, 22, and 31. Significantly greater inhibition (P < 0.05 to P < 0.00002) was observed with a combination of TA1-RTA and 131I-labeled 520C9 (100 μCi or 250 μCi) than with either single agent on days 10, 22, and 31.

days to 356 days. Biological variation was noted between the two studies, but when both experiments are considered together, survival of >180 days was observed in 8 of 12 mice and survival of >300 days was observed in 6 of 12 (Table 2).

**DISCUSSION**

This study documents a synergistic interaction between radionuclide conjugates and immunotoxins that target p185HER-2 both in cell culture and in vivo. Despite an extensive preclinical and clinical literature regarding conjugation of monoclonal antibodies with drugs, toxins, and radionuclides (reviewed in Ref. 27), we could find no reports that have considered the interaction of immunotoxins with radionuclide conjugates. Mechanisms underlying the synergistic interaction between radiation and immunotoxins are not fully defined. Observations presented above suggest that immunotoxins may interfere with radiation-induced DNA repair. Because RTA inhibits protein synthesis, the inhibition of repair could relate to the depletion of repair enzymes or peptide cofactors that have relatively short half-lives or that are in limiting concentration. Several studies have demonstrated that immunotoxins can induce apoptosis (28, 29), and this is true of anti-p185HER-2 RTA. The inhibition of protein synthesis alone generally does not induce apoptosis (30, 31), but the persistence of unrepaired DNA damage may contribute to the enhancement of programmed cell death after irradiation. In the present study, a 3-h treatment of clone-9002-18 cells with cycloheximide failed to potentiate the cytotoxicity of ionizing radiation. An RTA immunotoxin may, however, persist within cells for much longer intervals than cycloheximide and may irreversibly inhibit protein synthesis by deurination of ribosomal proteins.

Synergy may relate, in part, to the activation of the HER-2 kinase or to greater internalization of p185HER-2 by the binding of antibodies to two different epitopes. Neither 520C9 nor the TA1 antibody triggers a significant (2-fold) increase in phosphorylation of HER-2 (19). Moreover, our previous studies have demonstrated that receptor kinase activity is not required for internalization of antibody (32) or for immunotoxin-induced cytotoxicity (19). Approximately 30% of cell-bound TA1 or ID5 anti-HER-2 antibody can be internalized within 1 h regardless of the ability of the antibody to induce phosphorylation of receptors (13). Internalization could lead to more rapid dehalogenation that could actually decrease the therapeutic activity of the radionuclide conjugate. The antibody alone fails to produce synergy, which suggests that antibody-induced signaling is not likely to change radiosensitivity. Synergy is also observed with immunotoxin and external beam radiation, which suggests that the binding of antibodies to two different epitopes is not required.

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plus 131I-labeled 520C9 (250 μCi/mouse on day 5), TA1-RTA (50 μg/mouse on day 5, 6, and 7) plus 131I-labeled 520C9 (100 μCi/mouse on day 5), TA1-RTA (50 μg/mouse on day 5, 6, and 7) plus 131I-labeled 520C9 (250 μCi/mouse on day 5). Each group contained 6 mice. Survival was significantly prolonged relative to the diluent in all of the treatment groups (P < 0.01). The combination of TA1-RTA and 131I-labeled 520C9 (100 μCi or 250 μCi) prolonged survival to a greater degree than did either single agent (P = 0.004).

Whatever the mechanism of the observed synergy, the use of immunotoxins and radionuclide conjugates may exert greater antitumor activity in vivo. A synergistic inhibition of growth was observed in cell cultures at relatively high rates of external beam γ radiation (27–3904 rads/min) and in vivo with relatively low rates of delivered dose of high energy β particles (766–1916 rads/several days). Because of the multicellular range of 131I β particles, the bystander activity of radiation may help to eliminate cells with relatively poor expression of p185HER-2. Despite the probable importance of p185HER-2 for the induction and maintenance of malignant transformation, substantial heterogeneity has been observed in the expression of the receptor in different areas of the same tumor (33). The direct or bystander effects of radionuclide conjugates may also eliminate cancer cells that are inherently resistant to immunotoxins. Observations with heterografts suggest that more complete elimination of tumor cells can be attained with the two modalities, permitting long-term disease-free survival in settings in which either single modality is inadequate. Greater efficacy with a single course of treatment may be critical for the use of conjugates that contain highly immunogenic plant or bacterial toxins.

Because BALB/c mice do not express human p185HER-2, the present study does not permit assessment of toxicity that could potentially arise by the targeting of immunotoxins and radionuclide conjugates to a receptor that is expressed on some normal human tissues. Immunohistochemical studies have detected low levels of p185HER-2 in human skin and gastrointestinal mucosa with trace expression in a number of other organs (33). To the extent that toxicity related to specific binding is dose-related, synergistic interactions between immunotoxins and radionuclide conjugates may permit effective treatment with lower doses of each agent. Studies in cell culture suggest that anti-p185HER-2 will exert optimal toxicity against cells with >10^6 copies of p185HER-2 per cell and little, if any, toxicity against cells with 10^4 receptors (14). Ultimately, toxicity can be assessed preclinically only in primates that express p185HER-2 that can bind RTA-anti-p185HER-2 conjugates.

Ovarian cancer is a particularly attractive target for clinical trials of serotherapy with monoclonal antibodies and their conjugates. Ovarian cancer afflicts 25,000 women in the United States each year and causes some 14,500 deaths annually. Despite advances in surgery and chemotherapy, the cure rate has changed little during the last decade. In the short run, a majority of patients will respond to cytoreductive surgery followed by chemotherapy that includes a platinum compound and a taxane. Approximately 40% of patients with advanced ovarian cancer will have a complete clinical response documented at second-look operations. At least one-half of these individuals will, however, experience recurrence of ovarian cancer, generally within 3 years, and will subsequently die from their disease. In this setting, treatment with radionuclide conjugates and immunotoxins could be justified if evidence of activity in Phase II trials could be obtained. Results of randomized Phase III trials could be evaluated with a relatively short lead time.

In the present study, the impact of immunotoxins and radionuclide conjugates was assessed using s.c. tumor transplants where access of these agents to tumor cells was achieved through the intravascular space. Study of s.c. nodules permitted frequent and precise measurement of the impact of the different treatments on tumor size. Because the progressive growth of ovarian cancer requires neovascularization, the use of this s.c. model may reflect the outcome of disease at several different sites. The distinctive pattern of spread for epithelial ovarian cancer over the surface of the peritoneum predisposes, however, to recurrence within the abdominal cavity in a majority of cases. After an apparently complete clinical response to conventional therapy, microscopic deposits of tumor can remain on the peritoneal surface. Consequently, in future preclinical studies, radionuclide conjugates and immunotoxins will be evaluated also against i.p. tumor transplants.

ACKNOWLEDGMENTS

We greatly appreciate the editorial assistance of Adrienne Mattea.

REFERENCES


Fig. 6 Survival after treatment of clone-9002-18 xenografts with saline, TA1-RTA (50 μg/mouse on day 5, 6, and 7), 131I-labeled 520C9 (100 μCi/mouse on day 5), 131I-labeled 520C9 (250 μCi/mouse on day 5), TA1-RTA (50 μg/mouse on day 5, 6, and 7) plus 131I-labeled 520C9 (100 μCi/mouse on day 5), TA1-RTA (50 μg/mouse on day 5, 6, and 7) plus 131I-labeled 520C9 (250 μCi/mouse on day 5). Each group contained 6 mice. Survival was significantly prolonged relative to the diluent in all of the treatment groups (P < 0.01). The combination of TA1-RTA and 131I-labeled 520C9 (100 μCi or 250 μCi) prolonged survival to a greater degree than did either single agent (P = 0.004).


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