Inhibition of Human Tumor Growth by Intraperitoneal Immunotoxins in Nude Mice

Alexander Marks, David Ettensohn, Michael J. Bjorn, Melanie Lei, and Reuben Baumal

Banting and Best Department of Medical Research, University of Toronto, Toronto, Ontario, Canada M5G 1L6 (A. M., M. B.); Department of Pathology, Hospital for Sick Children, Toronto, Ontario, Canada M5G 1X8 (D. E., R. B.); and Cetus Corporation, Emeryville, California 94608 (M. J. B.)

ABSTRACT

Intracavitary administration of immunotoxins may play a role in the control of malignant effusions. Selection of immunotoxins for this form of therapy is based on their prior evaluation in preclinical studies. Monoclonal antibodies (mAb) 454A12 (antitransferrin receptor), and 260F9 are directed against antigens which are present on tumor cells in pleural and peritoneal effusions of patients with adenocarcinoma of the breast and ovary. In the present study, immunotoxins derived by conjugating these mAb to recombinant ricin A (rRA) were shown to be cytotoxic to human ovarian adenocarcinoma HEY cells in vitro and in vivo. In the in vitro assay 454A12-rRA and 260F9-rRA were 1000-fold and 10-fold, respectively, more cytotoxic than free rRA against HEY cells, and both immunotoxins were potentiated approximately 1000-fold by monensin. For in vivo studies HEY cells were injected i.p. into nude mice at a challenge dose (3 x 10⁶ cells) which produced carcinomatosis with ascites, leading to death 30 days following injection. Administration of 454A12-rRA i.p. following the challenge dose resulted in a complete cure, whereas administration of 260F9-rRA with monensin significantly prolonged survival. The greater cytotoxicity of 454A12-rRA than 260F9-rRA against HEY cells could be accounted for by the greater number of binding sites and higher internalization rate for 454A12-rRA and mAb 454A12 than 260F9-rRA and mAb 260F9, respectively.

These results suggest a potential role for 454A12-rRA and 260F9-rRA plus monensin in the intracavitary therapy of malignant effusions associated with carcinoma of the breast and ovary. In the case of 260F9-rRA, this represents the first preliminary indication of the suitability of this immunotoxin for intracavitary therapy of malignancies.

INTRODUCTION

Intracavitary administration of immunotoxins might be a useful therapeutic modality to contain the spread of malignancies confined to the pleural and peritoneal cavities. Monoclonal antibodies 454A12 and 260F9 have been raised against human breast tumor tissue and are directed against the human transferrin receptor and a M₉ 55,000 cell surface protein enriched on breast and ovarian cancer cells, respectively (1, 2). These mAbs react with variable proportions of tumor cells in malignant effusions obtained from patients with adenocarcinoma of the breast and ovary (3), but are not specific for these malignancies, since each also reacts with a number of normal human tissues and other tumor cell types (1). Immunotoxins derived by conjugating mAb 454A12 and 260F9 to rRA are among the most cytotoxic reported for human breast and ovarian tumor cell lines and primary cultures (2, 4-6). Although they are also cross-reactive with normal mouse tissues, was nonetheless effective in treating mice bearing syngeneic i.p. tumors to which it was targeted (7).

As a preliminary indication of the suitability of 454A12-rRA for intracavitary therapy, two groups have recently reported that the i.p. administration of this immunotoxin prolonged the survival of mice given injections of a lethal dose of human tumor cells (6, 8). No similar studies have been reported for 260F9-rRA. In order to examine the suitability of 260F9-rRA for intracavitary therapy and identify possible factors which influence its potency against target cells, we compared the cytotoxicities of 454A12-rRA and 260F9-rRA to human ovarian adenocarcinoma HEY cells in vitro and in vivo [i.e., growing i.p. in nude mice (9, 10)]. Our results suggest a potential role for 260F9-rRA and monensin in the intracavitary therapy of malignant effusions associated with carcinoma of the breast and ovary.

MATERIALS AND METHODS

Mice. Four- to 6-week-old female BALB/c nu/nu (nude) mice (Charles River Valley, St. Constant, Quebec, Canada) were used as hosts for tumors produced by i.p. administration of HEY cells.

Cell Lines. The cultured HEY cell line was derived from the human epithelial ovarian adenocarcinoma xenograft HX-62, as previously described (11). The HEY-1B cell line was a subclone which had been adapted to grow i.p. in nude mice (9). The cells were grown as monolayers in α-minimal essential medium (University of Toronto Media Centre, Toronto, Ontario, Canada) supplemented with 10% heat-inactivated fetal bovine serum, penicillin, streptomycin, and l-glutamine (all from GIBCO, Grand Island, NY) at 37°C in a humidified 5% CO₂ incubator, and were harvested by trypsinization.

Immunotoxins. The 454A12 and 260F9 mAb are murine IgG1 immunoglobulins (1). MOPC 21 is a murine IgG1 with no known binding specificity (12). Antibodies were reacted with iminohistamine and the resulting sulfhydryl group was activated by the addition of 5,5'-dithiobis(2-nitrobenzoic acid). This material was reacted with rRA (which has a single reactive cysteine) to form disulfide-linked mAb-rRA (13). The immunotoxins were analyzed by electrophoresis on 5 to 12% gradient polyacrylamide slab gels in the presence of sodium dodecyl sulfate. Scanning densitometry of a Coomassie blue-stained gel revealed that the preparations contained less than 3% of either unconjugated mAb or rRA. This analysis also showed that 24% of the 454A12-rRA preparation consisted of 1 rRA/mAb (1-mer), 35% of 2 rRA/mAb (2-mer), 20% of 3 rRA/mAb (3-mer), and the remaining 19% of higher-molecular-weight species. For the 260F9-rRA preparation, the corresponding proportions were 51%, 1-mer; 31%, 2-mer; 7%, 3-mer; and 5%, higher-molecular-weight species. A molecular weight of 200,000 was used to estimate molar concentrations. The 50% lethal dose for a single i.p. dose of either immunotoxin in 20-g female BALB/c nu/nu mice was approximately 180 μg (9 mg/kg).

Monensin. Monensin was purchased from Sigma Chemical Co. (St. Louis, MO). The drug was dissolved at 10⁻⁴ M in ethanol and then diluted 100-fold to a final concentration of 10⁻⁵ M in PBS for addition to culture medium or injection into mice.

Binding and Internalization Assays. HEY cell monolayers in 96-well
INHIBITION OF HUMAN TUMOR GROWTH BY IMMUNOTOXINS

flat bottomed culture plates (GIBCO) were incubated with varying concentrations of $^{125}$I-labeled mAb or immunotoxin, as previously described (14). To determine affinity constants and number of binding sites, the data were analyzed by using a modified Scatchard plot (14). Internalization assays were performed by determining the fraction of radioactive mAb or immunotoxin associated with the cells following a pH 2.5 wash as described in the literature (15, 16). The internalization rate constant ($K_i$) was determined as described (16).

**In Vitro Cytotoxicity.** Samples containing 2 x 10⁴ HEY cells in 200 µl were seeded in 96-well flat bottomed culture plates and grown for 2 days, at which time they formed confluent monolayers. The monolayers were then incubated at 37°C for 18 h with immunotoxin in 100 µl of α-minimal essential medium supplemented with 2% fetal bovine serum, in the presence or absence of 1 x 10⁻⁹ M monensin. Inhibition of protein synthesis by the immunotoxins was determined by adding 1 µCi of $[^{3}H]$leucine (600 Ci/mmol; New England Nuclear, Mississauga, Ontario, Canada) and incubating the cells for an additional 4 h. The cells were washed 3 times with incubation medium, solubilized with 2 N NaOH, and counted in a scintillation counter. The extent of inhibition of protein synthesis was estimated as the percentage of counts/min of $[^{3}H]$leucine incorporated in the presence of immunotoxin relative to incorporation in the absence of immunotoxin.

**Tumor Growth Inhibition.** Aliquots of 3 x 10⁴ HEY cells were injected i.p. into nude mice (10). One, 24, and 48 h later, the mice were given injections i.p. of 200 µl PBS containing 10 µg of immunotoxin and 1.4 µg ($1 \times 10^{-5}$) mmenonisin, and were monitored for the development of ascites and survival time. The presence of i.p. tumor nodules was assessed visually at autopsy and by histological examination of tissue sections of peritoneal tumors. All experiments involving mice were carried out according to a protocol approved by the Animal Care Committee of the Ontario Cancer Institute.

Other Methods. Protein concentration was determined by the method of Lowry et al. (17). The statistical significance of differences was determined by using a one-way analysis of variance and the Student-Newman Keuls multiple range test.

RESULTS

**Binding Characteristics of mAb and Immunotoxins to HEY Cells.** MAb 454A12 and 260F9 and their corresponding rRA immunotoxins had similar affinities for HEY cells (Table 1). The number of binding sites on HEY cells for mAb 454A12 and 454A12-rRA was approximately 1000-fold higher than that for mAb 260F9 and 260F9-rRA, respectively (Table 1). In the case of 454A12 there was a 2-fold decrease in affinity following conjugation of the mAb to rRA (Table 1). The internalization constant for mAb 454A12 and 454A12-rRA was 7-fold higher than for mAb 260F9 and 260F9-rRA (Fig. 1).

**Cytotoxicity of immunotoxins to HEY Cells in Vitro.** Inhibition of protein synthesis in HEY cells in vitro by 454A12-rRA and 260F9-rRA, and unconjugated rRA is shown in Fig. 2, and the corresponding concentration of immunotoxin required for 50% inhibitions of protein synthesis for these reagents are summarized in Table 2. In the absence of monensin, 454A12-rRA and 260F9-rRA were approximately 1000-fold and 10-fold, respectively, more cytotoxic to HEY cells than unconjugated rRA (Fig. 2; Table 2). In the presence of 10⁻⁷ M monensin, the cytotoxicities of 454A12-rRA and 260F9-rRA were potentiated 1000-fold and 2000-fold, respectively (Fig. 2; Table 2). The same potentiation was observed in the presence of 10⁻⁸ M monensin, but there was no potentiation in the presence of 10⁻⁹ M monensin (data not shown). Several control immunotoxins, consisting of mAb which did not bind to HEY cells conjugated to rRA were all less cytotoxic to HEY cells in vitro than unconjugated rRA. For example, the concentration

**Table 1 Binding characteristics of monoclonal antibodies and immunotoxins to HEY cells**

<table>
<thead>
<tr>
<th>Immunoglobulin</th>
<th>Immunoglobulin-rRA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoclonal antibody</td>
<td>$K_i$ ($M^{-1}$)</td>
</tr>
<tr>
<td>454A12</td>
<td>0.5 ± 0.5</td>
</tr>
<tr>
<td>260F9</td>
<td>3.6 ± 1.5</td>
</tr>
</tbody>
</table>

* Affinity constant of binding.
| Number of binding sites per cell.
| The values were obtained by analyzing data of binding assays by using a modified Scatchard plot (14). The values shown are means ± SEM of 3 independent assays. Each point in the binding assay was performed in triplicate.
of MOPC 21-rRA required for 50% inhibition of protein synthesis was 200 nM in the absence of monensin and 50 nM in the presence of monensin.

Tumorigenicity of HEY Cells. Tumorigenicity was assessed by i.p. injection of varying numbers of HEY cells into nude mice. A dose dependency was found between the number of cells injected and survival time, with survival times ranging from means of 30 to 65 days following injection of $3 \times 10^3$ to $3 \times 10^6$ cells, respectively (Fig. 3). Mice developed abdominal distention 2 to 3 weeks before death. At autopsy, 1 to 4 ml of ascitic fluid was present in the peritoneal cavity and tumor nodules were seen on the peritoneum, mesentary, diaphragm, and surface of the liver. Mice given injections of $3 \times 10^3$ HEY cells showed no evidence of i.p. tumor growth when sacrificed 100 days following injection of the cells.

Tumor Growth Inhibition. Mice were given injections i.p. of $3 \times 10^3$ HEY cells. One, 24, and 48 h later, the mice were given injections i.p. of PBS (control mice) or immunotoxins, either alone or together with monensin, and were monitored for survival (Table 3). Control mice developed ascites and died after approximately 30 days. Mice treated with 454A12-rRA alone, or 454A12-rRA and monensin, were sacrificed after 100 days and showed no evidence of tumor formation. Mice treated with 260F9-rRA alone and 260F9-rRA plus monensin, survived to mean times of 40 to 52 days, respectively. These survival times were significantly longer ($P < 0.05$) than that of control mice, and the addition of monensin prolonged survival significantly over treatment with 260F9-rRA alone ($P < 0.05$). No tumor-free mice were found among the mice whose survival had been prolonged by administration of 260F9-rRA. Treatment with unconjugated rRA and monensin did not prolong survival (Table 3). Similarly, treatment with unconjugated mAb 454A12 or 260F9 and monensin, or with monensin alone, did not prolong survival (data not shown).

**DISCUSSION**

A number of ricin A-containing immunotoxins which are potentially useful for therapy of adenocarcinoma of the breast and ovary have been identified on the basis of their binding and cytotoxicity to human breast and ovarian tumor cell lines *in vitro* (2, 4–6, 18). In most cases, there was a direct correlation between the effectiveness of binding of the immunotoxins to target cells and their respective cytotoxicities, although anomalies have been reported (4, 18). The reasons for these discrepancies are not clear. In order to examine this issue, we determined the binding and internalization characteristics of mAb 454A12 and 260F9, and their corresponding immunotoxins to HEY cells, and correlated these characteristics with the respective cytotoxicities of the immunotoxins. mAb 454A12 and 260F9 bound to HEY cells with a similar $K_a$ of about $5 \times 10^8$, but the number of binding sites for mAb 454A12 on HEY cells was approximately 4-fold higher than for 260F9 (*i.e.*, $8 \times 10^8$ *versus* $2 \times 10^8$). These binding characteristics were not markedly changed by conjugating the mAb to rRA. In the case of 454A12-rRA the $K_a$ was approximately 50% lower than that of unconjugated 454A12. In the protein synthesis inhibition assay *in vitro*, 454A12-rRA was 100-fold more cytotoxic than 260F9-rRA and both were potentiated approximately 1000-fold by monensin. The higher cytotoxicity of 454A12-rRA than of 260F9-rRA against these cells, can be accounted for by the greater number of binding sites and higher internalization rate for 454A12-rRA than for 260F9-rRA. Other factors such as the processing of these immunotoxins to release free rRA are also likely to play a role in determining their respective cytotoxicities. In this respect, the greater proportion of monomeric immunotoxin molecules in the 260F9-rRA than in the 454A12-rRA preparation should favor its cytotoxic potential (19), but is clearly not sufficient to compensate for the other properties which render 454A12-rRA a more effective immunotoxin.

The results of the *in vivo* tumor inhibition assay demonstrated the ability of 454A12-rRA to eliminate tumor cells from the peritoneal cavity of mice following the i.p. administration of this immunotoxin. Since $3 \times 10^3$ tumor cells were injected i.p., and the tumor burden had to be reduced to $3 \times 10^3$ or less to prevent tumor formation, our results indicate that there was a minimum of a 2-fold log kill with our treatment schedule. The *in vivo* potentiation of 454A12-rRA by monensin could not be evaluated, since treatment with 454A12-rRA alone resulted in tumor-free mice. Similar results were obtained independently with different treatment schedules.

In the case of 454A12-rRA our results confirm similar observations previously reported in two other nude mouse models of i.p. carcinomatosis (6, 8). In comparison with these tumor models the HEY tumor model has certain advantages. First, approximately 100-fold lesser number of HEY cells ($3 \times 10^3$) are needed to reproducibly cause diffuse i.p. carcinomatosis, ascites, and death at approximately 30 days. Second, the prolongation of survival correlates well with the decrease in the initial i.p. tumor challenge dose and is very sensitive to small

![Fig. 3. Tumorigenicity of HEY cells. Mice were given injections i.p. of various numbers of HEY cells and were monitored for survival. Points, means; bars, SEM; numbers above bars, number of mice.](image-url)
decreases in the initial tumor burden in the range of $3 \times 10^5$ to $3 \times 10^6$ cells (Fig. 2). Because of these features, the HEY tumor model promises to be a very useful in vivo assay system for comparing the relative cytotoxicities of immunotoxins, drug conjugates, and radioimmunoconjugates incorporating mAb reactive with HEY cells. We took advantage of the sensitivity of this system to demonstrate the cytotoxicity of 260F9-rRA against HEY cells in vivo. Although the prolongation of survival achieved with 260F9-rRA was less than that achieved with 454A12-rRA, this represents the first demonstration of cytotoxicity of 260F9-rRA against human tumor cells in a nude mouse model. In the presence of monensin, 260F9-rRA prolonged survival to an average of 52 days. This effect was specific to 260F9-rRA, since four other less potent anti-HEY cell rRA-containing immunotoxins administered by using identical treatment schedules failed to achieve the same tumor growth-inhibitory effect in the HEY tumor model (10). In the case of 8C-rRA and M2A-rRA a modest prolongation of survival to 45 and 40 days, respectively, was observed, while in the case of M2D-rRA and 10B-rRA, no prolongation of survival relative to PBS-injected controls was seen.

The failure of achieve a complete cure with 260F9-rRA may be due to the relatively poor sensitivity of HEY cells to 260F9-rRA. The reported cytotoxicity of 260F9-rRA to human breast and ovarian tumor cells lines and primary cultures has been found to be much higher than the cytotoxicity seen in the present study (2, 4, 5). This suggests that 260F9-rRA might be a more effective immunotoxin in clinical trials than could be predicted on the basis of our results in nude mice by using HEY cells. Furthermore, the greater selectivity of 260F9-rRA than 454A12-rRA for human breast and ovarian cell lines in vitro (4) is an additional consideration in evaluating these immunotoxins for clinical use, which might favor the choice of 260F9-rRA over 454A12-rRA. In either case, i.p. delivery should decrease the serum concentrations of both immunotoxins, thus minimizing toxicity to sensitive normal tissues.

The second issue addressed by using the HEY tumor model was the evaluation of the usefulness of monensin in potentiating immunotoxin action. Our results indicate that monensin enhanced the cytotoxicity of immunotoxins both in vitro (in the case of 454A12-rRA and 260F9-rRA), and in vivo (in the case of 260F9-rRA). The in vitro potentiation of ricin A-containing immunotoxins by monensin has been documented extensively in the past (13, 20, 21). However, the question of the in vivo effectiveness of monensin in mice is more relevant to its eventual clinical application. In this context, the method of preparation of the drug for administration to mice appears to be critical for its activity. For example, monensin conjugated to linoleic acid (8) or human serum albumin (22), or dissolved in dimethyl sulfoxide (23), has been shown to potentiate the cytotoxicity of ricin A-containing immunotoxins against human tumors disseminated i.p. (8) or systemically (22, 23), in nude mice. In our case, monensin was initially dissolved at $10^{-3}$ M in ethanol and then diluted 100-fold in PBS to a final concentration of $10^{-5}$ M for i.p. injection into mice, resulting in successful potentiation of immunotoxin action. In a previous study, in which monensin was dissolved at $10^{-2}$ M in ethanol prior to dilution with aqueous buffer for i.p. injection into mice, potentiation was not observed (8), possibly due to the insolubility of the drug at the step of the initial aqueous dilution from ethanol.

Our results indicate that partial elimination of disseminated human ovarian tumor cells from the peritoneal cavity of a mouse can be achieved by i.p. administration of an immunotoxin. With respect to relevance of these results to a potential clinical application, several clinical studies in ovarian cancer patients using i.p. administered radiolabeled mAb have suggested that elimination of free-floating tumor cell clusters from the peritoneal cavity, using immunoguided radiation as an adjunct to therapy of malignant ascites, might be a more realistic therapeutic goal than elimination of solid tumor nodules (24–26). Similar clinical studies with i.p. administered immunotoxins have not yet been reported. Because of the widespread reactivity of tumor cells in malignant effusions from patients with adenocarcinoma of the breast and ovary with mAb 260F9 (3), the demonstration of the potential suitability of 260F9-rRA and monensin for intracavitary therapy of malignant effusions is of clinical interest. A recent Phase I trial of i.v. administered 260F9-rRA in patients with metastatic breast cancer has suggested that this immunotoxin might be too toxic for systemic use (27). However, this does not preclude its potential usefulness for intracavitary administration. Continuing studies in the HEY tumor model will attempt to define further conditions for optimizing the effectiveness of 260F9-rRA against target tumor cells.

ACKNOWLEDGMENTS

We wish to thank Dr. L. L. Houston for critical review of the manuscript.

REFERENCES

15. Wargalla, U. C., and Reifseld, R. A. Rate of internalization of an immunotoxin...


Inhibition of Human Tumor Growth by Intraperitoneal Immunotoxins in Nude Mice

Alexander Marks, David Ettenson, Michael J. Bjorn, et al.


Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/50/2/288