Mediation of Reduction of Spontaneous and Experimental Pulmonary Metastases by Ricin A-Chain Immunotoxin 45-2D9-RTA with Potentiation by Systemic Monensin in Mice


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ABSTRACT

We developed a model to assess the therapeutic effects of the 45-2D9 ricin A-chain immunotoxin (RTA) on pulmonary metastases. The 45-2D9 mouse monoclonal antibody recognizes a M, 74,000 glycoprotein highly expressed by rat fibroblasts transformed with the Kirsten sarcoma virus (transformed rat fibroblasts). These cells metastasize spontaneously and form lung colonies in nu/nu and irradiated BALB/c mice. Injection i.v. of 45-2D9-RTA specifically reduced formation of spontaneous pulmonary metastases and lung colonies originating from freshly disaggregated tumor cells or cultured cells. Antibody alone or mixed with unconjugated ricin A chain and an immunotoxin that recognizes a melanoma-chain, which is less nonspecifically toxic than whole ricin, to specifically blocked the 45-2D9-RTA activity in vivo. Administration of the immunotoxin demonstrated highly specific toxicity by killing 3 to 4 logs of target cells in vitro. In this report, we describe development of a model of both experimental and spontaneous established metastases to test the efficacy of the systemically administered immunotoxin in vivo and to assess the effects of potentiating agents administered systemically.

INTRODUCTION

Monoclonal antibodies that recognize tumor cells selectively could potentially function as delivery vehicles for cell toxins. The A chain of the toxin ricin, derived from the castor bean, could potentially function as delivery vehicles for cell toxins. Previous reports of the therapeutic efficacy of immunotoxins for solid tumors have been limited to treatment of primary s.c. and i.d. tumors and to local-regional administration of immunotoxin (3-6). A model closely approximating the common clinical situation of established metastatic solid tumor would be useful for assessing immunotoxin efficacy for adjuvant therapy and provide a system for evaluating potentiating agents that may increase immunotoxin efficacy by altering host mechanisms responsible for inactivation of the toxin component.

Our laboratory has developed a mouse monoclonal antibody (45-2D9), which recognizes a M, 74,000 serine-phosphorylated glycoprotein that is expressed by some ras oncogene-transformed cells (7, 8). The 45-2D9 antibody also recognizes an epitope on human carcinomas with minimal expression by normal human tissues (8). An immunotoxin was developed by conjugating the A chain of the toxin ricin to the antibody. This monoclonal antibody recognizes a M, 74,000 glycoprotein expressed by rat fibroblasts transformed with the Kirsten sarcoma virus (transformed rat fibroblasts). These cells metastasize spontaneously and form lung colonies in nu/nu and irradiated BALB/c mice. Injection i.v. of 45-2D9-RTA specifically reduced formation of spontaneous pulmonary metastases and lung colonies originating from freshly disaggregated tumor cells or cultured cells. Antibody alone or mixed with unconjugated ricin A chain and an immunotoxin that recognizes a melanoma-chain, which is less nonspecifically toxic than whole ricin, to specifically blocked the 45-2D9-RTA activity in vivo. Administration of the immunotoxin demonstrated highly specific toxicity by killing 3 to 4 logs of target cells in vitro. In this report, we describe development of a model of both experimental and spontaneous established metastases to test the efficacy of the systemically administered immunotoxin in vivo and to assess the effects of potentiating agents administered systemically.

MATERIALS AND METHODS

Purification of Monoclonal Antibody 45-2D9. The 45-2D9 antibody was purified from either ascites or spent hybridoma supernatant. For ascites production, pristane-primed BALB/c mice were inoculated i.p. with 1 x 10^6 hybrids/mouse. The ascites were dialyzed against 0.14 M NaPO4, pH 7.8, and then centrifuged to remove debris. Ascites (70 ml total) or supernatant was then passed over a 1.5 x 30-cm column packed with a 40-ml bed of Protein A agarose (Sigma, St. Louis, MO) equilibrated with 0.14 M NaPO4. The column was then washed until the A280 returned to baseline. Bound immunoglobulin was eluted with pH steps of 8.0, 5.0, 4.5, and 3.5. The 45-2D9 antibody eluted at pH 4.5 without contaminating isotypes. The purity of the eluted antibody was monitored by two-dimensional SDS-PAGE under reducing conditions. The column was cleared with citrate buffer, pH 3.5, and reequilibrated with 0.14 M NaPO4, pH 8.0. Pooled fractions of antibody were dialyzed against DPBS, 200 v/v, with three changes at 24-h intervals, then aliquoted at 1 mg/ml, and lyophilized for storage. The isotype from each lot was routinely checked with reagents supplied in a murine immunoglobulin subtype identification kit (Boehringer Mannheim, Indianapolis, IN). Immunoglobulin concentration was determined by radial immunodiffusion.

Preparation of Immunotoxin. Ricin A-chain and the 45-2D9 immunotoxin were prepared by Xoma Corporation (Berkeley, CA). The technique of Kernan et al. (9) was used to purify the ricin A chain and synthesize the antibody-toxin conjugate. Ricin A-chain was purified by affinity column chromatography. Enzymatic activity was assayed as the ability to inhibit protein synthesis in a cell-free reticulocyte lysate system. All lots used for conjugate preparation must have values for an inhibitory concentration for a 50% decrease in protein synthesis of 1 to 5 x 10^-4 g/ml. The purification's purity was monitored by SDS-PAGE on 12.5% gels under nonreducing conditions and a contaminating band corresponding to native ricin was not detected.

Antibody-toxin conjugates were prepared by coupling ricin A chain to antibody molecules by a disulfide linkage. Antibody 45-2D9 (1 to 2 mg/ml) was dialyzed against 0.1 M NaPO4-0.1 M NaCl, pH 7.7, and a 15- to 20-fold M excess of N-succinimidyl-3-(2-pyridyldithio)propionate was added with vigorous mixing. After incubation at room temperature for 30 min, the solution was dialyzed against two changes of PBS. After dialysis, the pyridyl-dithiopropionate group-to-antibody ratio was determined as described by Carlson et al. (10). The ricin A-chain was reduced by addition of dithiothreitol to a final concentration of 50 mm, followed by incubation for 1 h at room temperature. The solution was dialyzed against PBS (4°C) to remove residual reducing agent. The ricin A-chain was concentrated using an Amicon stirred cell with a YM10 membrane to a final concentration of 4 mg/ml. Five- or 10-fold M excess ricin A-chain was added to the pyridyl-dithiopropionate-antibody solution and incubated for 16 h at 4°C.

Preparations of antibody conjugates were purified from free ricin A-chain by sizing chromatography using either G-150 or ACA 44 resins. The purified conjugate was then concentrated with an Amicon YM30...
IMMUNOTOXIN MEDIATION OF REDUCTION OF METASTASES

membrane and filter sterilized with a Millipore HV Millex filter (Millipore Products, Bedford, MA). The ratio of ricin A-chain to antibody was determined by a densitometric scan of SDS-PAGE of the different species of antibody-A-chain conjugates with calculation of the percentage of species and its A-chain number. The percentage of conjugated as compared with unconjugated antibody was determined by laser densitometric analysis (LKB 2202 Ultrascan) of an SDS-PAGE separation of the conjugate on a 7.5% nonreducing gel. Lots routinely contained more than 80% conjugates with a mean of two ricin A-chains/antibody molecule. The absence of B-chain was confirmed by identifying inhibitory activity of thymidine incorporation by TRF cells in the presence of 0.1 M galactose. The conjugates remained active for up to 3 months when stored at 4°C without added carrier protein. A control immunotoxin, IND-2-RTA, was prepared in a similar manner. This immunotoxin recognizes a human melanoma-associated antigen and inhibits tritiated thymidine incorporation by the 196 melanoma cell line. This antigen is not present on TRF cells. Immunotoxin potency was monitored for each new lot by inhibition of tritiated thymidine incorporation by the appropriate cell line as described below.

Inhibition of Thymidine Incorporation by Immunotoxin. To measure inhibition of tritiated thymidine incorporation, TRF cells or 196 melanoma cells were suspended in Dulbecco's modified Eagle's medium (Biofluids, Rockville, MD) and 2% fetal calf serum. Varying concentrations of antibody, immunotoxin, or ricin A-chain were added. All cultures were maintained in quadruplicate for each test group; total culture volume was 0.2 ml. Control cultures consisted of complete medium alone added to cells or medium without cells. Plates were incubated at 37°C in a humidified 8% CO₂ atmosphere for 72 h. Twelve to 18 h before termination of the test, the cultures were labeled with 0.5 μCi of tritiated thymidine (6 Ci/mM; New England Nuclear, Boston, MA) in 0.01 ml of PBS. The cultures were harvested using the PHD cell harvester (Cambridge Technology, Inc., Cambridge, MA). Samples were counted in a liquid scintillation counter. The degree of inhibition of thymidine incorporation was expressed as

\[
\% \text{ of control} = \frac{\text{cpm of cultures + experimental additive}}{\text{cpm of control cultures}}
\]

Inhibition was always statistically significant (P < 0.05) by the two-sided Student's t test when the cpm for cultures containing immunotoxin was less than 70% of the cpm for control cultures.

Cell Lines and Culture Conditions. Rat fibroblasts transformed with the Kirsten sarcoma virus and expressing the K-ras oncogene (TRF cells) express high levels of the gp74 antigen recognized by 45-2D9 (11). The TRF cell line is fully transformed by the following criteria: altered cellular morphology; focus formation in liquid culture; growth in soft agar; progressive growth as a lethal tumor in nu/nu mice; formation of pulmonary colonies after i.v. injection in nu/nu mice and in irradiated (5 Gy) BALB/c mice. We selected this cell line for therapy experiments because of its rapid growth rate, high level of antigen expression, and ability to form metastases from a relatively small inoculum. DNA hybridization studies with the 0.6-kilobase SalI-EcoRI fragment of the HiiH3 v-K-ras plasmid demonstrated strong specific hybridization for DNA extracted from TRF cells (12, 13). The biochemical characteristics of the antigen and the genetic characteristics of the transfected cell line will be discussed elsewhere (2). Cell line 196 is a human melanoma cell line that expresses the M, 97,000 protein melanoma-associated antigen and grows as a lethal tumor in nu/nu mice. All cell lines were tested for Mycoplasma and murine viruses PVM, Poly, GDVII, Ectro, K, Reo, Sendai, MHV, LCM, LDH, MAD, and were negative (Animal Health Diagnostic Laboratory, Frederick, MD). Cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% FCS, 2 mM L-glutamine, 2 mM sodium pyruvate, and 100 μg/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone (all from Biofluids). Cell lines were grown at 37°C in 8% CO₂ and air.

Preparation of Single-Cell Suspensions of Fresh Tumor Cells. Tissue culture cells were inoculated s.c. in 6- to 8-week-old female NIH nu/nu mice. Tumors grew to 1- to 2-cm diameters within 2 to 3 weeks and were excised under sterile conditions. The tumor was finely minced and then suspended in a solution of 0.1% collagenase (Type IV; Sigma), 0.01% hyaluronidase (Type V; Sigma), and 0.002% DNPase (Type I; Sigma) with continuous stirring overnight at room temperature. Tumor cells were then washed twice with complete medium and the debris and nonviable cells separated with density-gradient centrifugation using lymphocyte separation media (Litton Bionetics, Kensington, MD). Viability was determined by trypan blue exclusion. The cells were cryopreserved and stored in a liquid nitrogen freezer.

Animal Model and Evaluation of Results. The TRF cells and other transfectants in this series grew as s.c. tumors and lung colonies following i.v. injection in both nu/nu and irradiated (500 cGy) 6- to 8-week-old female BALB/c mice. Mice given injections in the tail vein of 5 × 10⁶ to 2 × 10⁷ TRF cells developed 150 to >250 lung colonies that were easily counted 10 to 12 days after injection. Metastases formed discrete white nodules on the blackened surface of the lung (insufflated with a 15% solution of India ink) when "bleached" with Fekete's solution (14). The injected tumor cells were derived from either a single-cell suspension of s.c. growing tumors cryopreserved in liquid nitrogen as described above or cells grown in tissue culture. Therapy was begun 2 days after tumor cell injection so that only established tumors were treated. The transplanted cells also spontaneously metastasized. Three weeks following injection of 5 × 10⁷ tumor cells into the hind footpad the tumor grew to 1 cm and amputation was performed. Two weeks later, the animals were killed. The animals' lungs were coded and randomly distributed. The metastases were then counted "blindly" by a second observer. Groups were compared using the Wilcoxon rank sum test. A P ≤ 0.05 was considered statistically significant. At the initiation of therapy, lung tumors were visible microscopically but not grossly (Fig. 1A).

Preparation and Administration of Potentiating Agents. Ammonium chloride was prepared as a stock solution of 10 mg/ml in DPBS, and 0.5 ml was administered i.p. 48 and 72 h following injection of tumor cells. A 2-fold increase in the dose resulted in more than 50% lethality for the mice. Chloroquine (Sigma) was prepared as a stock solution of 1 mg/ml in DPBS, and 0.5 ml was administered i.p. 48 and 72 h after injection of tumor cells. This is about 6-fold more than the dose administered to humans for malaria prophylaxis based on weight. Monensin was prepared in DMSO and administered i.p. in doses ranging from 0.03 to 33.0 μg/mouse in 0.1 ml DMSO 48 and 72 h following injection of tumor cells. Complete absorption of these doses was calculated to result in concentrations in the extracellular fluid space equivalent to or higher than concentrations that potentiate the toxicity of 45-D9-RTA in vitro. All solutions were filtered through a 22-μm filter before use.

RESULTS

Therapy of Spontaneous Metastases. The effect of 45-D9-RTA immunotoxin on the formation of spontaneous pulmonary metastases after amputation of a 1-cm footpad tumor of TRF cells was assessed. At the time of amputation, pulmonary metastases were not visible grossly, but microscopic tumor foci were present in the lung (Fig. 1A). At the time of sacrifice 2 weeks later, grossly visible metastases were present, generally numbering between 25 and 50 (Fig. 1B). Mice given injections i.v. of a single dose of 20 μg of 45-D9-RTA 2 days after amputation had significant reduction in pulmonary metastases compared with mice receiving either an equivalent dose of IND-2-RTA control immunotoxin or DPBS (Fig. 2). Reductions of 80 and 100% compared with both controls were achieved in two additional experiments (data not shown).

Therapy of Experimental Metastases. Although immunotoxin efficacy was demonstrated by the spontaneous metastasis model, the small number of metastases and their biological variability made it difficult to use the model in experiments...
Fig. 1, A, hematoxylin- and eosin-stained, formalin-fixed section of lung from a BALB/c nu/nu mouse given an injection of $2.5 \times 10^4$ TRF cells in the footpad 3 weeks before sacrifice. At this time point amputation is performed. A single metastasis is present in this section. B, lungs excised from BALB/c nu/nu mouse 2 weeks after amputation of a 1-cm TRF footpad tumor. The lungs were given injections of India ink so that the metastases appear white on the black background.

receiving multiple groups and large numbers of mice. We therefore developed an experimental metastasis model. Initially, TRF cells were inoculated s.c. in BALB/c nu/nu mice, and single-cell suspensions were prepared from the resultant tumors. The fresh tumor cells were injected i.v. and mice were treated with a single dose of 45-2D9-RTA 48 h following injection. A significant reduction in lung colony formation was observed after treatment with the immunotoxin in comparison with the saline and IND-2-RTA controls (Table 1, experiments 1 and 2). These results were comparable to those for the spontaneous metastasis model.

Immunotoxin Specificity. A third model system was developed using injection of cultured TRF cells. Injection i.v. of $2.5 \times 10^4$ cultured TRF cells resulted in the formation of 200 to 300 lung colonies when mice were sacrificed 10 days later. BALB/c mice were given injections of cells after 5 Gy of irradiation. Immunotoxin specificity was demonstrated in this model (Fig. 3). Mice receiving a single i.v. injection of 45-2D9-RTA had significantly reduced lung colony formation compared with that of mice receiving saline, 45-2D9 antibody, 45-2D9 antibody and unconjugated ricin A-chain with a molar concentration equivalent to the immunotoxin, or IND-2-RTA. Additional evidence for specificity was demonstrated by a competitive inhibition experiment (Fig. 4). Immunotoxin 45-2D9-RTA (100 µg) mixed with 500 µg of mouse immunoglobulin significantly reduced lung colony formation compared with IND-2-RTA. This was completely abrogated by mixing 45-2D9-RTA with 500 ng of unconjugated 45-2D9 antibody. Additional evidence for specificity was obtained in three experiments in which 45-2D9-RTA failed to reduce significant lung colonies formed by a NIH 3T3 c-Ha-ras tertiary transfectant that does not express the gp74 antigen (data not shown).

Effect of Potentiators in Vivo. Both ammonium chloride and chloroquine can potentiate the toxicity of 45-2D9-RTA by one log in vitro (7). The monovalent ionophore, monensin, also potentiates 45-2D9-RTA toxicity with a one-log increase in the
BALB/c mice (n = 10 to 15/group) received 5 Gy followed by tail vein injection of 5 × 10⁴ fresh TRF cells (experiment 1), 2.5 × 10⁴ fresh TRF cells (experiment 2), or 2.5 × 10⁴ tissue culture cells (experiments 3 to 8). Mice were given injections i.v. of immunotoxin and i.p. of monensin when indicated 48 h following tumor cell injection. An additional injection of monensin was given 72 h following tumor cell injection. DMSO was given as an i.p. control injection in mice not receiving monensin for experiments 4 to 8. All mice were sacrificed on day 10, and the lung colonies were counted (see "Materials and Methods"). The significance of differences between groups was determined by the Wilcoxon test.

### Table 1: Specific inhibition of lung colony formation by 45-2D9-RTA and potentiation of antitumor effect by i.p. monensin

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*p = 0.05 compared with all controls by Wilcoxon test.

**DISCUSSION**

Our study demonstrates that a systemically administered ricin A-chain immunotoxin can reduce established micrometastases from a solid tumor. Griffen et al. (15) have shown potentiation with esterified monensin in regional therapy of mesothelioma.

The therapeutic efficacy of immunotoxins has been demonstrated in several animal models (3–6, 16, 17). These models represent either hematological malignancies or primary tumors treated by local or regional administration of immunotoxin. The effectiveness of immunotoxin therapy for metastases from solid tumors remains an important question. New therapeutic approaches to metastases of the common solid tumors are needed, and the availability of several monoclonal antibodies with selectivity for tumors makes this an attractive strategy. Therefore we investigated the effectiveness of immunotoxin in three distinct models of metastatic solid tumor. The 45-2D9 monoclonal antibody and the TRF cells seemed appropriate for an animal model because the characteristics of these cells resemble known characteristics of human tumors. TRF cells are heterogeneous in the expression of the gp74 antigen because...
breast, colon, and lung. The TRF cells have a 3-day doubling time and grow from a small inoculum. They are also relatively resistant to natural-killer and lymphokine-activated cell-mediated lysis in vitro (18).

The efficacy of immunotoxin therapy was demonstrated in three different model systems of pulmonary metastases: spontaneous metastases; experimental metastases from fresh tumor cells; and experimental metastases from cultured tumor cells. The 45-2D9-RTA immunotoxin was effective in all models. The high level of toxicity of 45-2D9-RTA for the TRF cells of three to four logs in vitro was undoubtedly a contributing factor.

The immunotoxin was not equally effective in all models, and less than 20% of mice had no evidence of tumor at sacrifice. Several factors may have contributed to this. The tumor cells are heterogeneous for expression of the gp74 antigen. Although the cell cycle dependency of antigen expression appeared to be the major cause of this, and antigen-negative clones have not been identified, the rapid turnover of the tumor may result in the presence of a significant antigen-negative growth fraction of the tumor cell population during the time of an effective circulating concentration of immunotoxin. Tumor burden may also be related to immunotoxin efficacy. The percentage of reduction in metastases formation was greatest for models in which the control group had fewer metastases. Other mechanisms of escape from the ricin A-chain's toxicity may also be involved, and these are being investigated. The IND-2-RTA immunotoxin demonstrated toxicity for TRF cells in occasional experiments in vitro and in vivo. This may be attributed to the nonspecific toxic effects of ricin A-chain and nonspecific binding of immunonoconjugates to TRF cells. Previous studies have demonstrated absence of the antigen recognized by the IND-2 antibody on TRF cells (7).

Identification of potentiation methods for immunotoxins will be important if these are to become clinically useful. Some potentiating agents active in vitro have been described, but efficacy in vivo has not been demonstrated (3, 19–22). These agents increase lysosomal pH and thus may retard degradation of the toxin molecule (23). Monensin administered i.p. potentiated the immunotoxin activity modestly but significantly. The mechanism responsible for this has not been clearly defined. Because monensin is a monovalent ionophore, it may potentiate by altering lysosomal pH. Monensin also blocks intracellular transport in vesicles from the Golgi apparatus (24). Perhaps this activity explains the potentiating ability of monensin in vivo that was not seen with agents that potentiate only by their lysosomotropic action. Additional experiments are required to determine this. Modification of the monensin molecule to enhance stability and delivery to the cell may further enhance its effectiveness.

**REFERENCES**


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7 S. P. L. Leong et al., unpublished observations.


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