An Immunotoxin Cytotoxic for Breast Cancer Cells in Vitro

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ABSTRACT

A potent immunotoxin was formed by conjugating the murine monoclonal antibody 323/A3 to the A chain of ricin. The 323/A3 antibody recognizes an antigen expressed by most human breast cancers. When binding of 323/A3 was examined by enzyme-linked immunosorbent assay, three human breast cell lines displayed strong binding, whereas two human breast cell lines and three non-breast cell lines displayed little or no binding. When the cell lines were tested at a concentration of 0.1 μg/ml, those cell lines which displayed an abundance of antigen by enzyme-linked immunosorbent assay were most sensitive to the effects of the 323/A3 immunotoxin. On the other hand, cell lines which displayed little or no antigen by enzyme-linked immunosorbent assay were not inhibited by the immunotoxin at this concentration. Further examination of the effects of immunotoxin concentration on protein synthesis confirmed the sensitivity of those cell lines rich in the 323/A3 antigen over a broad dose range. Similarly, three cell lines which displayed little of the 323/A3 antigen demonstrated little inhibition of protein synthesis with various concentrations of 323/A3 immunotoxin. However, two cell lines which displayed little antigen were intermediate in their sensitivity to the 323/A3 immunotoxin. Preclinical evaluation of immunotoxins as potential therapeutic agents will require accurate and sensitive screening of a wide variety of cell types. The 323/A3 remains of interest in studying the effects of immunotoxin in a defined in vivo model system.

INTRODUCTION

The primary goal of chemotherapy in breast cancer, whether in the setting of primary or advanced disease, is to eliminate all malignant cells with minimal damage to normal tissues. Despite great advances in the therapy of breast cancer, this goal has yet to be reached. Current modalities of breast cancer chemotherapy suffer from two major limitations: lack of specificity and drug resistance. Both of these limitations could conceivably be addressed if it were possible to selectively target potent cell poisons to cancer cells.

The potential of antibodies to be the vehicle for targeted therapy has long been recognized (1). Several investigators have recently developed MAb reactive with breast cancer-associated antigens (2--22). To be useful in targeted therapy of breast cancer, however, a MAb must recognize antigens which allow highly selective binding to breast cancer cells and little or no binding with normal tissues. Because few antibodies have the capability of selectively distinguishing between breast tumor cells and their normal cell counterparts, there have been only a few reports of antibodies that were effective in targeted therapy of breast cancer cells (23, 24).

We have characterized one MAb 323/A3, which recognizes a large proportion of human breast cancer cells in addition to other human neoplasms and normal tissues (25). Subsequently strong binding was observed to 3 human breast cell lines (MDA-7, T47-D, and ZR75.1), suggesting that these cell lines are rich in the antigen recognized by 323/A3 (Fig. 1). Two human breast cell lines (MDA-231, HBL-100) and 3 non-breast cell lines displayed little or no binding, suggesting these cell lines were poor in this antigen. The finding that some cell lines expressed large amounts of the target antigen, whereas others did not, allowed us to examine the possibility of studying 323/A3 as an immunotoxin initially in vitro and later in an in vivo model. In this paper, we describe the preparation of an immunotoxin by conjugating the toxic A chain of ricin to 323/A3 and the selective cytotoxicity of the 323/A3 immunotoxin in vitro.

MATERIALS AND METHODS

Antibodies

The purification and characterization of 323/A3 and F5C (anti-ovalbumin) have been described. Briefly, 323/A3 is a mouse IgG1, monoclonal antibody which recognizes a M, 43,000 membrane glycoprotein found on the surface of human breast cells and other human tissues (25). F5C is a mouse IgG1 monoclonal antibody which recognizes ovalbumin (26).

Cell Cultures

MCF-7 Cells. MCF-7 human breast cancer cells were a gift of Dr. Herbert Soule (Michigan Cancer Foundation). MCF-7 contains both ER and PgR and was grown as previously described (27). Growth medium consisted of Eagle’s minimal essential medium supplemented with 6 ng/ml insulin, 2 mm L-glutamine (Gibco), 1% nonessential amino acids (Gibco), 10 mm HEPES buffer (pH 7.3), 0.2% sodium bicarbonate, 25 μg/ml gentamicin (Irvine Scientific), and 5% calf serum (K. C. Biologicals).

MDA-231. MDA-231 human breast cancer cells were obtained from E. G. and M. Mason Research Institute (Dr. Erling M. Jensen). MDA-231 are ER and PgR negative (28). Growth medium is the same as MCF-7 except supplemented with 10% fetal bovine serum (K. C. Biologicals).

ZR75.1. ZR75.1 human breast cancer cells are ER and PgR positive and were obtained from E. G. and M. Mason Research. Growth medium is the same as described for MDA-231. T47-D. T47-D human breast cancer cells are PgR positive and ER negative (28) and were obtained as a gift from Dr. Geoff Greene (Ben May Laboratory for Cancer Research). Growth medium was the same as described for MDA-231.

HBL-100. HBL-100 cells are receptor negative cells (28) derived from normal human milk epithelium. Growth medium consisted of McCoy’s Medium 5A supplemented with 2 mm L-glutamine, 0.2% sodium bicarbonate, 50 μg/ml penicillin-streptomycin (Gibco), and 10% fetal bovine serum.

K.B. K.B. oral epidermoid human cancer cells were obtained from American Type Culture Collection. Growth medium consisted of RPMI 1640 medium supplemented with 2 mm L-glutamine, 1 mm sodium pyruvate, 0.2% sodium bicarbonate, 50 μg/ml penicillin-streptomycin, and 10% fetal bovine serum.

WI-38, WI-38 VA 13 (subline 2RA) human lung fibroblasts were obtained from American Type Culture Collection. These are virus-transformed cells, and growth medium consisted of BME in Hanks’ balanced salt solution (Gibco) supplemented with 1% BME-amino acids, 1% BME vitamins (Gibco), 0.2% sodium bicarbonate, 10 mm HEPES buffer (pH 7.3), 25 μg/ml gentamicin, and 10% fetal bovine serum.
Isolation of Ricin A Chain

With 2 nM -ululimiinc. 10 HIMHEPES buffer (pH 7.3), 0.2% sodium
methylation as described by Krolick et al. (29). The A chain was then further
purified by passage over an asialofetuin-Sepharose column to remove
Bimbo. Growth medium consisted ofDulbecco’s modified Eagle’s medium (Irvine Scientific) supplemented
with 20% FBS:PBS. •, MCF-7; O, ZR75.1; V, T47-D; O, K.B.; A, MDA231; X, HBL100;
&D, WI-38; B, 3T3.

Balb/3T3. 3T3 mouse embryo fibroblasts (clone A31) were obtained
from American Type Culture Collection. Growth medium consisted of
Dulbecco’s modified Eagle’s medium (Irvine Scientific) supplemented
with 2 mm L-glutamine, 10 mm HEPES buffer (pH 7.3), 0.2% sodium
bicarbonate, 25 mg/ml gentamicin, and 10% calf serum.

All the above cell lines were cultured in Corning plastic culture flasks
(75 cm²) at a density of 1 × 10⁴ cells/flask in appropriate growth
medium and grown in a humidified 5% CO₂ incubator at 37°C.

Isolation of Ricin A Chain

Ricin (RCAS₃) was purchased from Vector Labs, Burlingame, CA. Ricin A chain was purified by reduction and ion exchange chromatography as described by Krolick et al. (29). The A chain was then further purified by passage over an asialofetuin-Sepharose column to remove B chains contaminating the preparation. Ricin A chain purified in this way routinely had 50% lethal dose in mice greater than 150 ng.

Antibody-Ricin A Chain Coupling

A stock solution of SPDPr (Pharmacia Fine Chemicals, Uppsala, Sweden) was prepared in dimethylformamide at a concentration of 10 mg/ml (30 mM). The antibody to be coupled was prepared as a 1-mg/ml (10 μg/ml) solution in pH 7.0 PBS (150 mM NaCl, 50 mM phosphate). Five μl of stock SPDPr was added per ml of antibody solution (SPDPr/antibody molar ratio = 25). The reaction mixture was incubated for 30 min at room temperature and then dialyzed for 60 min against 1 liter of PBS. The antibody-SPDPr conjugates were separated from uncoupled A chains by gel filtration on a 1 x 50-
cm Sephacryl S-400 column (Pharmacia Fine Chemicals, Uppsala, Sweden) in pH 7.0 PBS. The conjugates were then filtered sterilized immediately.

Enzyme-linked Immunosorption Assay

Cell lines were tested for the presence of the 323/A₃ antigen by ELISA using a modification of the method of Layton and Smithyman (30). Cells were plated in 96-well plates (Costar) at a density of 5 × 10⁴ cells/well and allowed to grow for 24 to 48 h to form a confluent monolayer. Growth medium was removed, and the cells were washed by a 30-s rinse (with shaking) in 50 μl/well of PBS containing 0.1% Tween 20. Supernatant was removed, and the cells were dried by incubation of the plates without covers for 30 min at 37°C. The cells were fixed by a 5-min incubation at room temperature with 50 μl of 70% methanol/3% hydrogen peroxide. Fixative was removed, and cells were rehydrated by incubation for 20 min at room temperature with 200 μl/well PBS. Plates were washed 2 times with PBS and incubated for 1 h at 4°C with 200 μl/well of 1% bovine serum albumin:PBS. The 1% BSA:PBS was removed, and the wells were then incubated overnight at 4°C with either 50 μl of hybridoma supernatant or purified 323/A₃ (or control) antibody diluted in 1% BSA:PBS. Plates were then washed 3 times with PBS and incubated for 3 h at room temperature with 150 μl of goat anti-mouse IgG:peroxidase (Cappel) diluted 1:5000 with 1% BSA:PBS. Following incubation with goat anti-peroxidase antibody, plates were washed 5 times with PBS and incubated for 10 min at room temperature with 150 μl of enzyme substrate (2.5 mg/ml O-phenylene-
diamine/0.05% H₂O₂) prepared in citric acid buffer (50 mM citric acid monohydrate/0.2 M sodium phosphate dibasic, pH 4.9). The reaction was stopped by addition of 50 μl of 1 M sulfuric acid to each well. Plates were then read in a Dynatech MR-600 ELISA reader at wavelength of 490 nm.

SDS-Polyacrylamide Gel Electrophoresis

Immunotoxins and unconjugated antibodies were separated by SDS-
PAGE under nonreducing conditions on a vertical slab gel apparatus as originally described by Laemmli (31) and modified by Wykoff et al. (32), using a separating gel matrix of 7.5% acrylamide/0.25% bisacryl-
amide (Bio-Rad) and a stacking gel of 3% acrylamide/0.25% bisacyr-
amide. For staining of gels with 0.5% Coomassie Blue R250, gels were
fixed in 10% acetic acid/40% methanol and destained in 7% acetic acid.

Cytotoxicity Assay

Since the primary mechanism of ricin intoxication involves the inhibition of protein synthesis, immunotoxin effects on target cells were monitored as a function of radiolabeled amino acid incorporation into protein. Cells were plated in growth medium in 96-well microtiter plates at a concentration of 3 × 10⁴ cells per well. The cells were incubated in triplicate sets of wells for 24 h at which time 150 μl of medium were added containing unconjugated or toxin-conjugated 323/
A₃ (or control FSC₉) antibodies at various concentrations. Following a 20-min incubation at either 4°C or 37°C, the cells were carefully washed 3 times with PBS and cultured for an additional 24 h in growth medium. At this time, 150 μl of leucine-free medium (Gibco, Grand Island, NY) containing 1 μCi/ml [³H]leucine (New England Nuclear, Boston, MA) were added, and the cells were incubated an additional 6 to 8 h at 37°C. The cells were then harvested and lysed using a Titertek cell harvester (Flow Labs, Rockville, MD). The cell lysates were collected onto glass fiber filters (Flow Labs, Rockville, MD) which were then counted in Ready Solve scintillation fluid (Beckman). Cytotoxicity data were plotted as the percentage of control protein synthesis (no immunotoxin
treatment) as assessed by [³H]leucine incorporation (ordinate) versus
concentration of conjugate (abscissa).

Animals

Four-wk-old female ovariectomized, homozygous inbred BALB/c-
nu/nu athymic mice were purchased from Harlan-Sprague-Dawley (Madison, WI). Bilateral ovariectomy was performed at 3 wk of age. Mice were housed in filtered laminar air flow hoods in standard vinyl cages with air filter tops. Cages, bedding, and food were autoclaved before use. Water was autoclaved, acidified to pH 2.8, and provided ad libitum.

Cell Inoculation

Logarithmically growing MCF-7 cells were harvested by scraping in
complete culture medium. Eight × 10⁴ cells were treated with 0.2 ml
of a 0.1-μl/ml concentration of either 323/A₃ or FSC₉ immunotoxin.
After a 20-min incubation at 37°C, the cells were washed with PBS and
resuspended in 0.2 ml of culture medium. The cells were inoculated s.c. on the flank immediately caudal to the axilla through a 22-gauge needle tunneled 1 cm to prevent leakage of the cell inoculation. Mice were supplemented with 17 β-estradiol (Innovative Research, Rockville, MD) as described by Osborne et al. (33).

RESULTS

Antibody-ricin A Chain Characterization. Immunotoxins were formed by combining the SPDP-derivatized MAb and the reduced A chain of ricin, allowing disulfide exchange to occur. Excess free A chain was then separated from the conjugation mixture by chromatography over a Sephacryl S-400 column. Following chromatography, fractions were precipitated with 50% saturated ammonium sulfate and analyzed on a 7.5% SDS-PAGE under nonreducing conditions.

The immunotoxins were consistently collected in Fractions 45 to 55. PAGE analysis demonstrated that each fraction does not contain a homogeneous molecular species but rather heterogeneous mixtures of uncoupled antibody and immunotoxin. Furthermore, antibody-ricin conjugates generally contained 1 to 2 ricin A chain molecules per molecule of antibody. Despite this heterogeneity, the levels of cytotoxicity did not vary among individual fractions containing immunotoxin as determined by PAGE. Unconjugated A chain was effectively removed by the Sephacryl S-400 chromatography and was usually collected in Fractions 75 to 85.

Immunotoxin Cytotoxicity in MCF-7 Cells. MCF-7 cells grown in 96-well plates were treated for 20 min with serial 1:4 dilutions of the 323/A3 or control (F5C4) immunotoxin. Additional controls for these experiments included unconjugated 323/A3, unconjugated F5C4, free ricin A chain, and PBS. Each dilution was tested in triplicate, and each experiment was repeated at least 3 times.

The 323/A3 immunotoxin was effective at completely inhibiting protein synthesis in MCF-7 cells using immunotoxin concentrations as low as 0.1 µg/ml (Fig. 2). At lower doses, this effect was rapidly lost. Thus, control levels of protein synthesis were observed at a concentration of 0.0015 µg/ml. In contrast, similar concentrations of unconjugated 323/A3 produced no inhibition of protein synthesis, although some inhibition was noted at very high doses (approximately 250 to 1000 times greater than those giving optimal killing by the toxin-conjugated antibody. Furthermore, an immunotoxin prepared with the irrelevant F5C4 (anti-ovalbumin) was approximately 1000-fold less toxic to MCF-7 cells than the 323/A3 immunotoxin. Free ricin A chain mixed with unconjugated 323/A3 did not significantly inhibit protein synthesis in MCF-7 cells.

Immunotoxin at a concentration of 0.1 µg/ml was incubated with 8 X 10⁶ MCF-7 cells for 20 min, followed by determination of cellular protein synthesis as described in "Materials and Methods." Fig. 2. Cytotoxicity of immunotoxins or controls for the human breast cancer cell line, MCF-7. •, 323/A3 immunotoxin; O, unconjugated 323/A3; □, F5C4 immunotoxin; △, unconjugated ricin A chain; □, 323/A3 plus unconjugated A chain. Dilutions of each immunotoxin or control were incubated with 5 X 10⁶ MCF-7 cells for 20 min, followed by determination of cellular protein synthesis as described in "Materials and Methods."
IMMUNOTOXIN CYTOTOXIC FOR BREAST CANCER

Our purpose was to use an antibody that reacted with human breast cancer tissue but not to mouse tissue, so that the variables encountered in immunotoxin therapy could be studied in detail in a nude mouse model. The MAb 323/A3 recognizes a 43,000 glycoprotein expressed in high density on the cell surface of 59% of human breast cancers examined as well as other human neoplasms and normal tissues (25). A panel of eight cultured cell lines was examined for binding by 323/A3 in a fixed cell ELISA (Fig. 1). Three breast cancer lines were found to be rich in the target antigen, whereas little or no binding was observed in the remaining five cell lines. Based upon this information, our goal was to demonstrate that an immunotoxin formed by linking 323/A3 to the A chain of ricin would cause differential cytotoxicity for those cell lines rich in the antigen when compared to those lines lacking the antigen. In other words, the 323/A3 immunotoxin should be less toxic or non-toxic to those cell lines in which antigen could not be detected.

The 323/A3 immunotoxin was extremely cytotoxic when tested on MCF-7 cells (Fig. 2). Protein synthesis as measured by [3H]-leucine incorporation was completely inhibited with concentrations as low as 0.1 μg/ml. The effect of the 323/A3 immunotoxin on MCF-7 cells was specific as neither an irrelevant immunotoxin (F5C4-ricin A chain) nor unconjugated A chain inhibited protein synthesis. Further, the exposure time necessary to maximize the cytotoxicity was 20 min. This is a relatively short exposure when compared to other reported breast tumor immunotoxins (24). The kinetics of immunotoxin binding, internalization, A chain release, and killing has not been defined in these in vitro mammary systems. The understanding of these kinetics in vitro, as well as the pharmacology of the immunotoxins in vivo, will be important in the selection of immunotoxins for use in humans.

The assay used in this study to determine cytotoxicity examined the degree of inhibition of protein synthesis. Even though the MCF-7 cells failed to exclude trypan blue and did not grow when recultured, it could be argued that the cells might have survived in vivo by mechanisms not active in vitro. Additionally, if immunotoxin treatment in vitro failed to effect cell death, there might be little reason to pursue the evaluation of such an immunotoxin in vivo. For these reasons, we attempted to rescue in vitro-treated MCF-7 cells by injecting them into nude mice. The MCF-7 cells can regularly be grown as solid tumors in 3-wk-old ovariectomized nude mice, reaching a size of about 1.5 cm³ in 2 wk (33). When the MCF-7 cells were treated with the 323/A3 immunotoxin at a concentration that produced 90% inhibition of protein synthesis in vitro, no tumors formed in vivo (Table 1). When the cells were treated with the same concentration of F5C4 immunotoxin or with PBS, six and eight tumors formed, respectively, in groups of ten mice. This experiment examines the possibility of in vivo rescue of immunotoxin-treated cells and was not intended to identify the precise degree of cytoreduction achieved in vitro. It does, however, suggest that further examination of in vivo therapy of established tumor microfoci will be of interest.

The 323/A3 immunotoxin was also effective at inhibiting protein synthesis in two other cell lines rich in the 323/A3 antigen. Although the ZR-75.1 and T47-D cell lines were not as sensitive to the effects of the immunotoxin as the MCF-7 cell line, all three target cell lines displayed greater than 90% inhibition of protein synthesis a concentration as low as 0.39 μg/ml. In contrast, the 323/A3 immunotoxin was not toxic to WI-38, K.B., and MDA-231, three cell lines which did not bind 323/A3 by ELISA. The minimal degree of protein synthesis inhibition at higher doses of 323/A3 immunotoxin was nonspecific and regularly seen with unconjugated antibodies at these concentrations.

The ELISA failed to correlate with the cytotoxicity observed in two cell lines, HBL-100 and 3T3. These cell lines did not bind 323/A3 as determined by ELISA (Fig. 1) and were not sensitive to the 323/A3 immunotoxin when treated with a single concentration of 0.1 μg/ml (Fig. 3). However, when cytotoxicity was examined over a wide range of concentrations, it was discovered that both cell lines were intermediate in their sensitivity to the immunotoxin (Fig. 4). The effect of the 323/A3...
immunotoxin appeared to be specific as neither the F5C4, immunotoxin or free ricin A chain inhibited protein synthesis in these cell lines.

The observation, that the HBL-100 and 3T3 cell lines are sensitive to the effects of the 323/A3 immunotoxin but fail to bind 323/A3, as determined by ELISA, points to the fact that routine binding analysis may be inadequate in predicting the usefulness of an antibody as an immunotoxin. There are a number of possible explanations for the discrepancy between observed binding and cytotoxicity. Perhaps the 323/A3 antigen is present in these cell lines below the level of detection by ELISA or immunofluorescence. As some investigators have postulated that only one molecule of ricin A chain need enter a cell to effect cell death (34, 35), it is conceivable that the cytotoxicity assay with the 323/A3 immunotoxin is more sensitive than other assays in detecting low levels of the antigen. As we have identified the antigen recognized by 323/A3 in the epithelium of human colon (25), it should not be surprising that this tumor-associated antigen might be found in other cell types in low levels. Further, just as different cell types may vary in degree of antigen expression, it is likely that they also differ in degree of antigen modulation and in the kinetics of immunotoxin-mediated cell killing. In any case, preclinical evaluation of immunotoxins as potential therapeutic agents probably requires the screening of a wide variety of cell types for toxicity.

In summary, the 323/A3 monoclonal antibody appears to recognize an antigen which is highly expressed by most human breast cancers. Once conjugated to the A chain of ricin, a potent immunotoxin is formed that displays in vitro cytotoxicity dependent on the expression of the 323/A3 antigen by various cell lines. Further, 323/A3-positive MCF-7 cells treated with the immunotoxin cannot be rescued by injection into nude mice. We intend to further examine the efficacy of this immunotoxin in treating established tumors in nude mice in order to identify how such immunotoxins might be used to treat human breast cancer.

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REFERENCES

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