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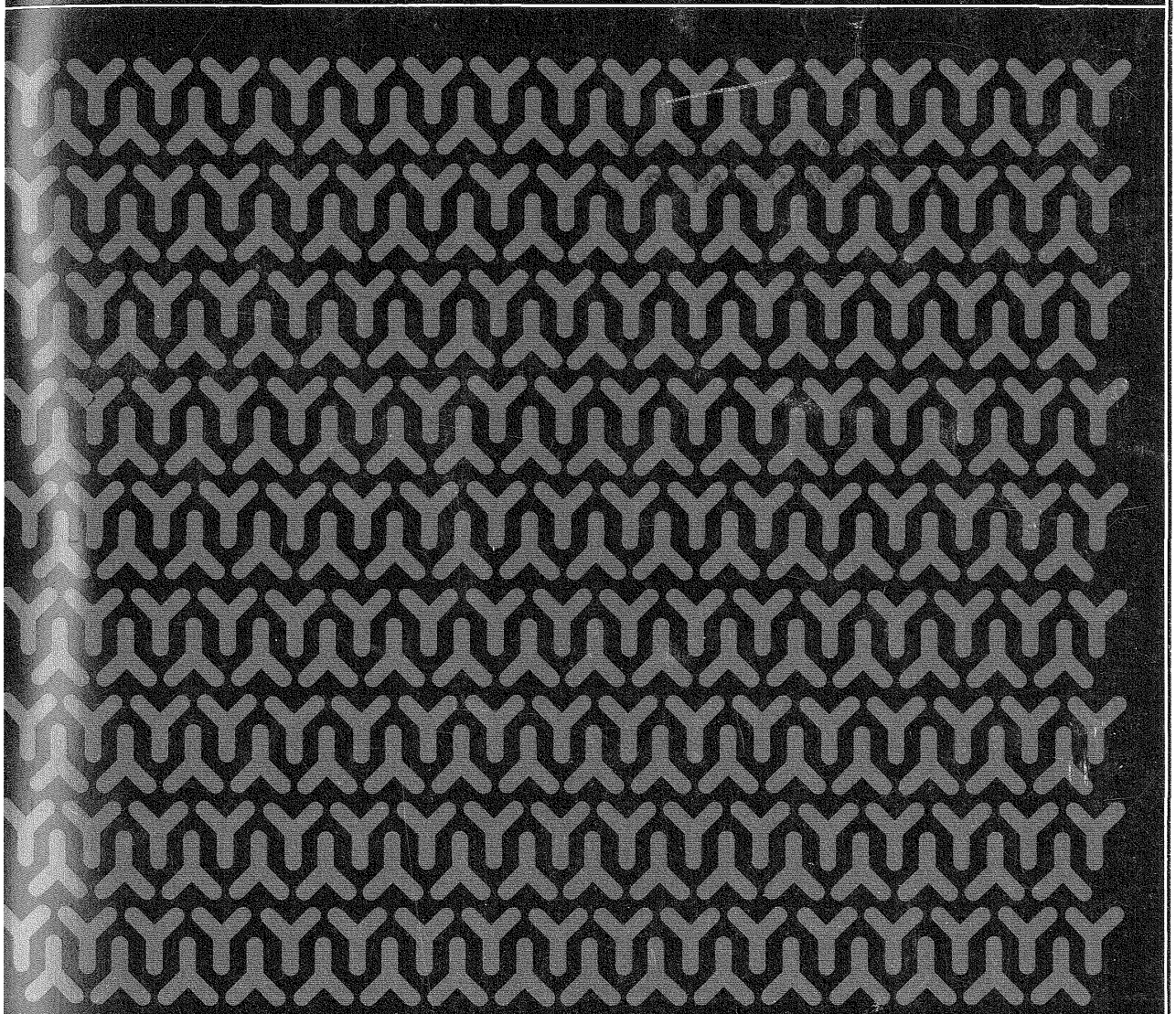

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Papers

A specific and potent immunotoxin composed of antibody ZME-018 and the plant toxin gelonin

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Murine monoclonal antibody ZME-018 recognizes a 240 Kda glycoprotein present on the surface of most human melanoma cells and on over 80% of human biopsy specimens tested. Gelonin is a ribosome-inactivating plant toxin similar in nature and rivaling the activity of ricin A chain. ZME-018 was coupled to purified gelonin using the reagents SPDP and 2-iminothiolane. The ZME-gelonin conjugate was purified by S-300 Sephacryl and Blue Sepharose chromatography, removing unreacted gelonin and antibody, respectively. PAGE analysis showed that ZME was coupled to 1, 2, or 3 gelonin molecules. The ZME-gelonin conjugate was 10^6-fold more active than gelonin itself in inhibiting the growth of log-phase human melanoma cells in culture. The immunoconjugate was not cytotoxic to antigen negative T-24 (human bladder carcinoma) cells. Treatment of melanoma cells with recombinant IFN-α or TNF substantially augmented the cytotoxicity of the immunoconjugate while treatment with IFN-γ had a minor effect. Using the human tumor colony assay of melanoma cells obtained from fresh biopsy specimens, >90% growth suppression was observed in 2 of 4 samples tested at a concentration of 250 ng/ml. In addition, 25% growth suppression was observed with a third sample tested, and no growth suppression was observed in 1 sample. Thus, clonogenic melanoma cells are sensitive in vitro *to the cytotoxic activity of this immunotoxin at concentrations which we presume are pharmacologically relevant.*

Keywords: Human melanoma; immunotoxins; gelonin; human tumor colony assay; cytokines.

Introduction

Since the introduction of monoclonal antibody technology, numerous efforts have been made to exploit the specificity of these reagents for cancer therapy.[1–5] As a first step, radiolabeled monoclonal antibodies to tumor cell surface antigens have been utilized successfully to image tumors in patients by external scintigraphy.[6,7] Extensive studies with antibodies to melanoma antigens[8–10] and to CEA[11,12] among others[13,14] have demonstrated specific tumor localization in man after systemic and intraperitoneal administration. Unfortunately, the accumulation of antibody by normal organs (*i.e.*, non-tumor) remains a key problem.

Because of their unique ability to localize within human tumors after systemic administration, antibodies have the potential to serve also as targeting vehicles for specific delivery of cytotoxic chemotherapeutic agents, toxic peptides, biological response modifiers, and radionuclides. Antibody ZME-018 (subclass IgG_{2A}) is reactive with epitope "a" of a 240,000 molecular weight antigen (gp240) found on the surface of over 80% of melanoma cell lines and fresh tumor samples.[15] Labeled with (^{111}In) and administered systematically to patients with melanoma, antibody ZME-018 was found to localize in 77% of soft tissue melanoma nodules.[16]

Several investigators have been interested in utilizing monoclonal antibodies as carriers of extremely active protein toxins.[17–19] In the case of these immunotoxins, the antibody serves as a vehicle for delivery of the toxin to the tumor. The binding of the antibody to the cell-surface target and pinocytosis or internalization of the antibody also serves as the mechanism for specific intracellular entry of the toxin.[20] The first clinical trials with immunotoxins utilized an antibody against colorectal carcinoma cells and ricin A chain (RTA) or diphtheria toxin A chain.[21,22] Since then, a variety of immunotoxins have been developed utilizing toxins such as abrin, ricin, RTA, diphtheria toxin, gelonin, and pseudomonas exotoxin.[23–26]

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The plant toxin gelonin is extracted from the seeds of the *Gelonium multiflorum* plant and exists in nature as a single polypeptide chain with a molecular weight of 29 KDa.[27] While the A chain of ricin (RTA) has been popular for use in immunotoxins,[28,29] gelonin, one of the 60 S ribosome inactivating hemitoxins, has several advantages over RTA. Gelonin appears to be more stable to chemical and physical treatment than RTA.[30] Furthermore, gelonin itself does not bind to cells and is, therefore, non-toxic (except in high concentrations) and is safe to manipulate in the laboratory. While the primary sequence of gelonin is unknown, mechanistic studies indicate that it operates identically to that of RTA.[31,32]

We have utilized the antimelanoma antibody ZME-018 as a model delivery vehicle for the plant toxin gelonin. The ZME-gelonin conjugate was synthesized, purified, and tested in a variety of *in vitro* human tumor models to determine its potential utility as an immunotherapeutic agent.

Materials and Methods

Materials

Antibody ZME-018 was prepared at Hybritech, Inc. (LaJolla, CA, USA), using salt fractionation and DEAE chromatography and was judged homogenous by SDS PAGE.[33] SPDP reagent (N-succinimidyl 3-[2-pyridyldithio] propionate), Sephacryl S-300 gel permeation resin and Blue Sepharose CL-6B resin were purchased from Pharmacia, Piscataway, NJ, USA. 2-Iminothiolane HCl was purchased from Pierce Chemical Co., Rockford, IL, USA. All materials were of reagent grade or higher. For estimation of the cell-free protein synthesis inhibitory activity of the toxin, a rabbit reticulocyte translation kit was purchased from Bethesda Research Labs (Bethesda, MD, USA).

Methods

Coupling of ZME-018 to gelonin. A stock solution of SPDP (6 mg/ml) in dry DMF was prepared. To 1 ml of a PBS solution containing 1 mg of ZME-018 in a 12 × 75 mm glass test tube, SPDP was slowly added to a 5-fold molar excess (approx. 10 μl of stock solution). The mixture was vortexed every 5 minutes for 30 minutes at room temperature.

Excess unreacted SPDP was removed from the sample by gel filtration chromatography on a Sephadex G-25 column (1 × 24 cm) pre-equilibrated in 100 mM sodium phosphate buffer pH 7.0 containing 0.5 mM EDTA (Buffer A). Fractions (0.5 ml) were collected and analyzed for protein content using the Bradford dye binding assay.[34] Absorbance (600 nm) was monitored in a 96-well plate using a Bio-TEK Microplate autoreader. Antibody eluted at the void volume (fractions 14–20) and these fractions were pooled and kept at 4°C.

For these studies, gelonin toxin was extracted from the seeds of *Gelonium multiflorum* and purified to homogeneity utilizing the method of Stirpe *et al*.[27] One milligram of purified gelonin (2 mg/ml PBS) was added to triethanolamine hydrochloride (TEA/HCl) buffer to a final concentration of 60 mM TEA/HCl and adjusted to pH 8.0. The solution was made 1 mM EDTA. 2-Iminothiolane stock solution (0.5 M in 0.5 M TEA/HCl pH 8.0) was added to a final concentration of 1 mM and the sample was incubated for 90 minutes at 4°C under nitrogen gas.

Excess 2-iminothiolane reagent was removed by gel filtration on a column of Sephadex G-25 (1 × 24 cm) pre-equilibrated with 5 mM bis-tris acetate buffer pH 5.8 containing 50 mM NaCl and 1 mM EDTA. Fractions were analyzed for protein content in 96 well microtiter plates using the Bradford dye binding assay. Gelonin eluted at the void volume (fractions 14–20). SPDP-modified antibody ZME was mixed with an equal weight of 2-iminothiolane modified gelonin. This proportion corresponded to a 5-fold molar excess of gelonin as compared to antibody. The pH of the mixture was adjusted to 7.0 by the addition of 0.5 M TEA/HCl buffer (pH 8.0), and the mixture was incubated for 20 hours at 4°C under nitrogen. Iodoacetamide (0.1 M in H_2O) was added to a final concentration of 2 mM to block any remaining free sulfydryl groups, and incubation was continued for an additional hour at 25°C.

Purification of ZME-gelonin complexes. To remove low molecular weight products and non-conjugated gelonin, the reaction mixture was applied to a Sephacryl S-300 column (1.6 × 31 cm) previously equilibrated with PBS. Fractions (1.0 ml) were collected and 50 μl aliquots were analyzed for protein content using the Bio-Rad dye binding assay. To remove unconjugated ZME-018, the high molecular peak (Fractions 28–41) from the S-300 column was applied to an affinity chromatography column of Blue Sepharose CL-6B (1 × 24 cm) pre-equilibrated with 10 mM phosphate buffer (pH 7.2) containing 0.1 M NaCl. After sample loading, the column was washed with 50 ml of buffer to elute completely non-conjugated antibody. The column was eluted with a linear salt gradient of 0.1 to 2 M NaCl in 10 mM phosphate buffer pH 7.2. Protein content of the eluted fractions was determined by the dye-binding assay described previously.[34]

Cell culture methods. Human bladder carcinoma (T-24), human cervical carcinoma, or human metastatic melanoma tumor cells A375M or AAB-527 were maintained in culture using minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum plus 100 μM non-essential amino-acids, 2 mM L-glutamine, 1 mM sodium pyruvate, vitamins, and antibiotics. Cultured cells were screened routinely and found free of mycoplasma infection.

Cell proliferation assay. Cell lines were maintained in culture in complete medium at 37°C in a 5% CO_2-humidified air incubator. For assays with combinations of TNF, immunotoxins, rIFNαA, and rIFNγ, cultures

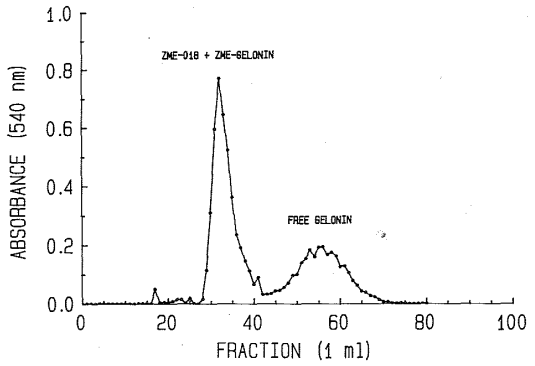

Figure 1. Purification of ZME-gelonin by S-300 gel permeation chromatography. High molecular weight material consisting of unmodified ZME and ZME-gelonin conjugate was eluted in fractions 28–41. Free gelonin eluted in fractions 45–65.

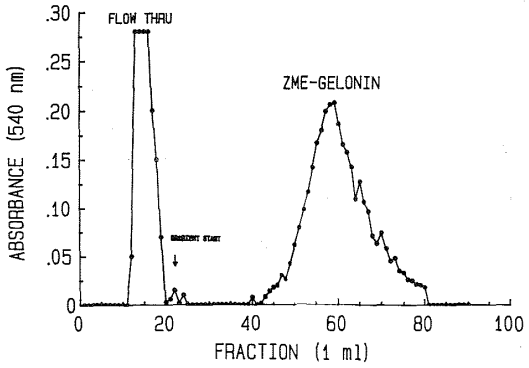

Figure 2. The high-molecular weight material from S-300 chromatography was applied to a column of Blue Sepharose and eluted with a linear salt gradient (0–300 mM NaCl). Two protein peaks were demonstrated: a flow-through peak (fractions 11–25) and a bound peak eluted with high salt (fractions 44–80).

were washed, detached using versene, and resuspended in complete medium at a density of 25 × 10^3 cell/ml. Two hundred µl aliquots were dispensed into 96-well microtiter plates and the cells were then allowed to adhere. This results in a sparsely seeded population of cells. After 24 hours, the media were replaced with media containing different concentrations of either immunotoxin, gelonin, TNF, IFNγ, or IFNα. The cells were incubated for 72 hours and analyzed for relative cell proliferation by crystal violet staining.

Crystal violet staining. Cells were washed 3 times with PBS containing calcium and magnesium fixed and stained with 20% (v/v) methanol containing 0.5% (w/v) crystal violet. Bound dye was eluted with 150 µl of Sörensen's citrate buffer (0.1 M sodium citrate, pH 4.2–50% (v/v) ethanol) for 1 hour at room temperature. The absorbance was measured at 600 nm using a Bio-Tek microplate reader. Relative cell proliferation (RCP) was calculated as follows:

$$\text{RCP} = \frac{\text{Mean Absorbance (Drug Treated)}}{\text{Mean Absorbance (Non-drug Treated)}} \times 100\% \quad \text{[eq 1]}$$

Human tumor colony assay. Tumor biopsy specimens were obtained from melanoma patients during clinically indicated biopsy procedures. Portions of tumor not required for standard diagnostic evaluation were transferred promptly to the human tumor cloning laboratory, wherein tumor cell suspensions were prepared aseptically.[35] Additionally, the A375P melanoma and the CEM leukemia cell lines from the American Type Culture collection (Rockville, MD, USA) were also studied. Testing for the effects of ZME-gelonin on the fresh melanoma cell suspensions and cell lines was assessed in the HTCA using standardized procedures for tumor cell plating in semi-solid medium (agarose) in the presence of complete medium containing 10% fetal calf serum. Each 0.5 ml culture plate contained 100,000 cells from fresh tumors and 10,000 cells from the cell lines.[36–38] ZME-gelonin prepared as described above was tested by addition to the culture plates shortly after tumor cell plating. ZME-gelonin was added to triplicate plates at each of four concentrations 0.025 ng/ml to 250 ng/ml. In addition to untreated control plates, unconjugated ZME-018 monoclonal antibody and free gelonin were tested in parallel. Cell lines and tumor cell cultures were incubated for an average of 10 days at 37°C in 5% CO_2 in air in a humidified incubator. Colony formation was evaluated with a viability stain[39] and an automated image analysis instrument optimized for colony counting.[40] Percent survival of ZME-018-treated cultures in relation to untreated controls were determined in the same experiments. Dose-response curves were then plotted graphically.

Results

Conjugation and purification of ZME-gelonin immunotoxin

Both ZME-018 and gelonin were modified by exposure to the reagents SPDP and 2-iminothiolane, respectively. Modification of ZME with 2 to 3 molecules of SPDP was accomplished by addition of a 6-fold molar excess of SPDP. Analysis of the number of SPDP molecules was accomplished spectrophotometrically.[41] The derivitization of ZME-018 and gelonin and the coupling were varied to provide optimal yield of ZME-gelonin conjugate (data not shown). *Figure 1* demonstrates separation of the ZME-gelonin conjugate from gelonin. Blue sepharose CL-6B chromatography (*Figure 2*) was effective at separating free ZME from ZME-gelonin conjugate. As demonstrated in *Figure 3*, the final eluent from Blue Sepharose was free of unreacted gelonin and ZME-018. The final product contained ZME-coupled to 1, 2, and 3 gelonin molecules. Average gelonin content was 1.5 molecules per antibody molecule. Average yield of purified im-

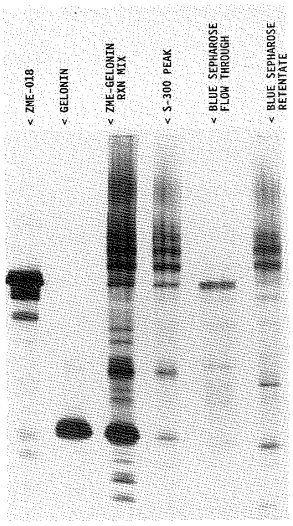

Figure 3. Silver stained PAGE analysis of ZME-gelonin conjugation and purification procedure.
LANE 1—ZME-018 antibody standard.
LANE 2—Native gelonin.
LANE 3—Unpurified ZME-gelonin reaction mixture showing the presence of unreacted ZME antibody and unreacted gelonin. Also shown are high molecular weight conjugate bands corresponding to ZME + 1 gelonin molecule, ZME + 2 gelonin molecules and ZME + 3 gelonin molecules.
LANE 4—High molecular weight peak from S-300 chromatography demonstrating removal of low molecular weight components including most of the free gelonin.
LANE 5—Flow-through fraction from application of the conjugate to a Blue Sepharose column. All of the unreacted antibody eluted from the column in the low-salt wash.
LANE 6—Final purification product eluted from the Blue Sepharose column. As shown, there was no remaining free antibody and only small amounts of unreacted gelonin in the eluted fraction.

munotoxin was approximately 20% of expected maximal value.

Binding of ZME-gelonin to cells in culture

The binding of ZME-gelonin immunotoxin to antigen positive (AAB-527 cells) or antigen negative (T-24 cells) was tested by ELISA assay. As shown in *Figure 4*, both native ZME and the ZME-gelonin conjugate bound well to target cells after 60-minute exposure. Surprisingly, the ZME-gelonin conjugate bound target cells better than did the native antibody. This increase was not due to modification of the antibody by SPDP since SPDP-modified ZME behaved identically to that of native ZME. The increase was also not due to binding of target cells to the gelonin portion of the molecule since pre-treatment of target cells with native gelonin had no effect on either antibody or immunotoxin binding. Thus, these results suggest that coupling of ZME to gelonin may affect antibody affinity for the gp240 antigen.

Neither ZME nor ZME-gelonin bound to antigen

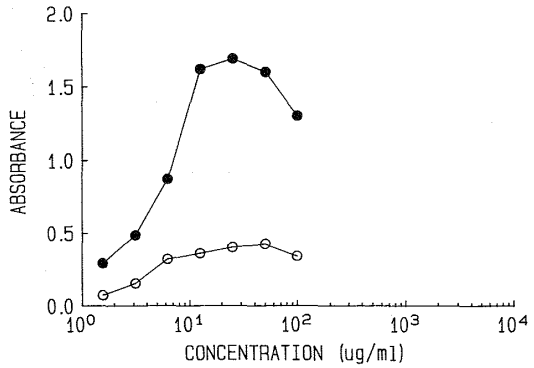

Figure 4. Comparative ELISA Assay of ZME (○) and ZME gelonin (●). Various concentrations of either ZME or ZME-gelonin were added to antigen-positive (AAB-527) human melanoma cells and incubated at room temperature for 60 minutes. The cells were washed and a standard ELISA was performed for murine antibody.

negative T-24 cells as estimated by ELISA assay (data not shown). Therefore, the apparent change in antibody affinity resulting in the coupling of ZME to gelonin does not appear to reduce selectivity of the antibody.

Cytotoxicity of ZME-gelonin

Cytotoxicity studies of the ZME-gelonin conjugate were performed on antigen-positive cells after continuous (72-hour) exposure to the immunotoxin or native gelonin. As shown in *Figure 5*, 50% cell death by the immunotoxin occurred at a concentration of approximately 0.02 μg/ml. A similar effect (i.e., 50% cell death) was observed for native gelonin at a concentration of 5 μg/ml. These concentrations are equated to a dose of 0.1 nM for ZME-gelonin and a dose of 100 nM of free gelonin to achieve reduction of the number of viable cells to 50% of control value.

The activity of both ZME-gelonin immunotoxin and free gelonin were determined using a cell-free translation assay system.[42] One unit of activity in this assay was defined as the amount of protein required to provide 50% inhibition of protein synthesis compared to untreated controls. Utilizing this assay, the specific activity of both the native gelonin and the ZME-gelonin conjugate were determined to be 2×10^8 μ/mg and 8.2×10^5 μ/mg, respectively. Target cells then were treated with various concentrations of ZME-gelonin and gelonin alone. As shown in *Figure 6*, a 50% inhibitory concentration was obtained using 50 units/ml of ZME-gelonin conjugate while 1×10^7 units/ml of the free gelonin were required to achieve the same effect.

The effect of ZME-gelonin was determined against antigen-negative T-24 cells in log-phase culture. Gelonin alone produced 50% cytotoxicity in these cells at a concentration of 10 μg/ml; similar to that found on AAB-527 melanoma cells (data not shown). However, the ZME-gelonin immunotoxin was not active against

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