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A potent and specific immunotoxin for tumor cells expressing disialoganglioside GD_2*

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Summary. Monoclonal antibody 14G2a (anti-GD_2) reacts with cell lines and tumor tissues of neuroectodermal origin that express disialoganglioside GD_2. mAb 14G2a was coupled to the ribosome-inactivating plant toxin gelonin with the heterobifunctional cross-linking reagent *N*-succinimidyl-3(2-pyridyldithio)propionate. The activity of the immunotoxin was assessed by a cell-free translation assay that confirmed the presence of active gelonin coupled to 14G2a. Data from an enzyme-linked immunosorbent assay demonstrated the specificity and immunoreactivity of the 14G2a-gelonin immunotoxin, which was identical to that of native 14G2a. Assays for complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) revealed that these functional properties of the native 14G2a antibody were also preserved in the 14G2a-gelonin immunotoxin. The gelonin-14G2a immunotoxin was directly cytotoxic to human melanoma (A375-M and AAB-527) cells and was 1000-fold more active than native gelonin in inhibiting the growth of human melanoma cells in vitro. The augmentation of tumor cell killing of 14G2a-gelonin immunotoxin was examined with several lysosomotropic compounds. Chloroquine and monensin, when combined with 14G2a-gelonin immunotoxin, augmented its cytotoxicity more than 10-fold. Biological response modifiers such as tumor necrosis factor α and interferon α and chemotherapeutic agents such as cisplatinum and *N*,N′-bis(2-chloroethyl)-*N*-nitrosourea (carmustine) augmented the cytotoxicity of 14G2a-gelonin 4- to 5-fold. The results of these studies suggest that 14G2a-gelonin may operate directly by both cytotoxic efforts and indirectly by mediating both ADCC and CDC activity against tumor cells; thus it may prove useful in the future for therapy of human neuroectodermal tumors.

Key words: Monoclonal antibodies – Toxins – Immunotoxins – Cytotoxicity

Introduction

There has been increasing interest in evaluating the potential of monoclonal antibodies (mAbs) that recognize a variety of tumor-associated antigens for the diagnosis and therapy of cancer. Murine mAbs directed against tumor cell-surface antigens have potential to localize within tumors selectively after systemic administration [7, 8, 13, 14]. A number of clinical trials that were conducted to evaluate the therapeutic efficacy of such mAbs per se have shown relatively limited antitumor effects [3, 10], whereas such studies with radiolabeled antibodies have shown more pronounced antitumor effects [3].

Immunotoxins are cytotoxic agents constructed by covalently linking mAbs directed against tumor-associated antigens with bacterial toxins e.g. *Pseudomonas* exotoxin [22] or to such plant toxins as ricin, abrin or gelonin [11, 23, 24, 26, 27, 34, 35, 37, 40]. Extensive clinical trials with immunotoxins have yet to be carried out, but immunotoxins do have the potential for use in adjuvant tumor therapy.

Gelonin is a single-chain glycoprotein of 28 kDa that acts by inhibiting cellular protein synthesis in a manner similar to that described for the A chains of ricin, abrin, and modeccin [32]. Studies on the mechanism of cytotoxicity of these toxins suggest that these proteins inactivate the eukaryotic ribosomes by cleaving a single-N-glycosidic bond at A-4324 of 28S rRNA in the ribosomes [4, 5]; this does not appear to involve co-factors, and occurs with an efficiency that suggest that single-chain toxins such as gelonin act enzymatically [33]. Gelonin by itself lacks the ability to bind to the cell surface and internalize into the cell [33]. However, once coupled to mAbs that bind to cell-surface antigens that are internalized, gelonin-containing immunotoxins evoke specific and impressive cytotoxicity [32]. Gelonin has several advantages over ricin A chain. Immunotoxins constructed with gelonin have been reported to localize better within tumors than ricin A immunotoxins [32]. Also, in some murine xenograft models, gelonin immunotoxins exert more potent in vivo therapeutic effects [32] than ricin A chain immunotoxins.

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mAb 14G2a directed against disialoganglioside GD_2 reacts with cell lines and tumor tissues of neuroectodermal origin [16]. This antibody was selected from the parental clone designated 14.18 by flow cytometry [17]. The antibody alone has some clinical utility in the treatment of patients with neuroblastoma (A. Yu, personal communication; R. Handegretinger, personal communication), which correlates well with previous in vitro data indicating that it effectively mediates antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) against tumor cells that express GD_2 [16].

The purpose of this study was to investigate whether the biological activity of this murine mAb could be further augmented by coupling to a plant toxin such as gelonin. In this report, we describe the generation, purification (of an immunotoxin composed of mAb 14G2a and gelonin), and the characterization of its in vitro activity against a variety of human tumor cell lines.

Materials and methods

Materials. CM-52 ion-exchange resin was purchased from Whatman Chemical Co., Clifton, N. J. The rabbit reticulocyte lysate kit and Cibacron-blue – Sepharose were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. 2-Iminothiolane and *N*-succinimidyl-3(2-pyridyldithio)-proprionate (SPDP) Sephadex G-25, and Sephacryl S-300 HR reagents were obtained from Sigma Chemical Co., St. Louis, Mo. Ethylene diaminetetraacetic acid (EDTA) was purchased from Boehringer Manneheim, Indianapolis, Ind. Triethanolamine hydrochloride was obtained from Kodak Chemical Co., Rochester, N. Y., and dimethyl-formamide was purchased from Aldrich Chemical Co., Milwaukee, Wis. Trizma hydrochloride was obtained from Bio-Rad Laboratories, Richmond, Calif.

Cell lines. The cultured human melanoma cell line A375-M was a gift from Dr. I. J. Fidler of M. D. Anderson Cancer Center, Houston, Tex. The human melanoma cell line AAB-527 was kindly provided by Dr. Pat Trown, Xoma Corporation, Davis, Calif. The T-24 bladder carcinoma cell line was purchased from the American Type Culture Collection, Rockville, Md. The NMB-7 (human neuroblastoma) cell line was obtained from Dr. Ralph A. Reisfeld, Research Institute of Scripps Clinic, La Jolla, Calif.

mAbs. mAb 14G2a is an isotype switch variant of mAb 14.18 (IgG_3) that recognizes disialoganglioside GD_2. This antibody was initially produced by two of the authors (K. Mujoo and R. A. Reisfeld) at Scripps Clinic and Research Foundation. Purified 14G2a mAb was also purchased from Brunswick Biotechnetics Inc., San Diego, Calif.

Purification of gelonin toxin. The gelonin toxin was essentially purified as described previously [33] with slight modifications. Seeds (*Gelonium multiflorum*) were obtained from United Chemical Co., Calcutta, India.

Coupling of mAb 14G2a and plant toxin gelonin. The mAb 14G2a was coupled to plant toxin gelonin using heterobifunctional cross-linking reagent SPDP as described earlier [12] using slight modifications. The detailed procedure is described elsewhere [29].

Purification of gelonin-14G2a conjugate. The coupled reaction mixture was first applied to a Cibacron-blue-sepharose CL-6B column to remove unconjugated antibody. The column was washed with 30 ml 10 mM sodium phosphate buffer (pH 7.2) containing 0.1 M NaCl. The gelonin-antibody conjugate bound to the column was eluted with 2 M NaCl phosphate buffer (pH 7.2). The protein content of the eluted fractions was determined by the Bio-Rad assay. To remove high-molecular-mass aggregates and free gelonin, the immunotoxin was then applied to a Sephacryl S-300 gel filtration column. The elution pattern was confirmed by electrophoretic analysis of pooled fraction on a 5%–15% gradient, non-reducing polyacrylamide gel. The fractions containing pure immunotoxin were pooled and stored at 4° C.

ELISA assay. Enzyme-linked immunosorbent assays (ELISA) were performed to compare the immunoreactivity of the immunotoxin with that of mAb 14G2a and to determine any binding of 14G2a-gelonin conjugate to non-target cells. The detailed procedure of ELISA has been previously described [16].

Translation assay. The rabbit reticulocyte in vitro translation assay was performed as described previously [1] to determine the activity of gelonin as well as that of the 14G2a-gelonin immunotoxin.

Cytotoxicity assay. Tumor cells, 2.5×10^3 (AAB-527) or 4×10^3 (A375-M melanoma cells) and 4×10^3 T-24 (bladder carcinoma cells) in complete media were plated in 96-well microtiter plates for 24 h. Following this, various concentrations of antibody, antibody-toxin conjugate, and free toxin were added and the cells incubated for an additional 72 h. The plates were then washed thoroughly with phosphate-buffered saline and were stained with 0.1% crystal violet in 20% methanol for 5–10 min. After staining, the plates were washed three times with distilled water and the dye was extracted by adding 200 µl Sorenson's buffer (0.1 M sodium citrate, pH 4.2, 50% v/v ethanol).

Effects of lysosomotropic drugs, biological response modifiers and chemotherapeutic drugs on the cytotoxicity of 14G2a-gelonin immunotoxin. The A375-M melanoma cell line was exposed to ammonium chloride (10 mM), chloroquine (40 µM), monensin (100 nM), as well as tumor necrosis factor (TNFα) (10 U/ml), interferon (IFNα) (10000 U/ml), carmustine (*N,N'*-bis(2-chloroethyl)-*N*-nitrosourea; BCNU) (15 µg/ml), and cisplatinum (0.05 µg/ml) simultaneously with various concentrations of immunotoxin and the effect of these agents on immunotoxin cytotoxicity was monitored by cytotoxicity assay as described above.

Complement-dependent cytotoxicity. Neuroblastoma cells (NMB-7) were suspended in 1 ml medium with 100 µCi ^{51}Cr (New England Nuclear) for 2 h at 37° C. The detailed procedure of this assay is described elsewhere [16]. The percentage specific lysis in this assay was calculated as:

$$\frac{(\text{experimental } ^{51}\text{Cr release}) - (\text{spontaneous } ^{51}\text{Cr release})}{(\text{maximal } ^{51}\text{Cr release}) - (\text{spontaneous } ^{51}\text{Cr release})} \times 100$$

Antibody-dependent cellular cytotoxicity. Tumor cells were labeled by the addition of 300 µCi ^{51}Cr in 1 ml RPMI-1640 medium for 2 h at 37° C. This method has also been described earlier [16].

Scatchard plot analysis. Melanoma cells (10^5 A375-M and AAB-527) were plated in 24-well plates and allowed to adhere overnight on 37° C. The cells were washed with cold medium and incubated with various concentrations (1–60 nM) of ^{125}I-labeled mAb 14G2a or 14G2a-gelonin immunotoxin for 2 h at 4° C. The non-specific binding was determined by the presence of a 200-fold excess of unlabeled antibody. After the 2-h incubation, the cells were washed three times with cold medium containing 1% bovine serum albumin and lysed with 0.1 M NaOH containing 0.1% sodium dodecyl sulfate. All analyses were performed in duplicate in a gamma scintillation counter, and data were analyzed by the method of Scatchard [30] for affinity constant and number of binding sites.

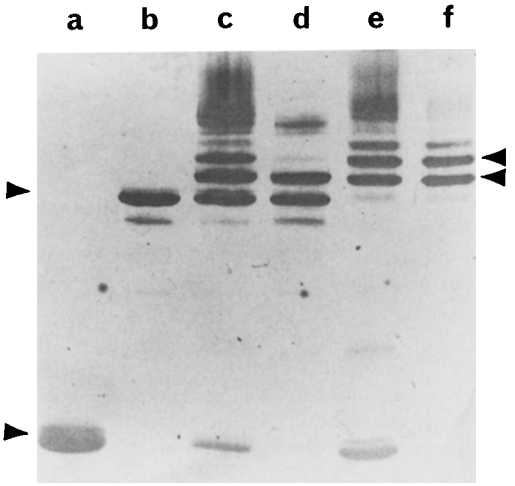

Fig. 1. Silver-stained, non-denaturing sodium dodecyl sulfate/polyacrylamide gradient gel (5% – 15%) of 14G2a-gelonin purification. *a* Gelonin. *b* Monoclonal antibody 14G2a. *c* Reaction mixture showing the free mAb 14G2a, free gelonin and mAb 14G2a coupled to one, two or three molecules of gelonin. *d* Blue-Sepharose wash – showing the presence of free antibody and monoconjugate. *e* Blue-Sepharose elution profile demonstrating the presence of free gelonin and conjugated antibody. *f* Elution profile of immunotoxin from S-300 gel permeation column free of high-molecular-mass aggregates and free gelonin

Results

Covalent conjugation of monoclonal antibody 14G2a and plant toxin gelonin

Gelonin toxin, extracted from the seeds of *Gelonium multiflorum* and purified as described earlier [33], is shown to be more than 97% pure from silver-stained polyacrylamide gels following electrophoresis under non-reducing conditions. Results of the cell-free translation assay [1] estimated the activity of the toxin at 2×10^8 units/mg protein, where 1 arbitrary unit of activity is the amout of protein required to reduce the translation assay to 50% of the control level [1]. The electrophoretic pattern of the reaction mixture purified on a Cibacron-blue – Sepharose and S-300 gel filtration column indicates that mAb 14G2a (Fig. 1, lane b) is bound to either one or two gelonin molecules (Fig. 1, lane f).

Immunoreactivity and specificity of 14G2a-gelonin immunotoxin

The immunoreactivity and specificity of 14G2a-gelonin immunotoxin determined by ELISA were indicated when 14G2a-gelonin immunotoxin bound to melanoma A375-M target cells with somewhat higher intensity than mAb 14G2a alone (Fig. 2). The specificity of 14G2a-gelonin immunotoxin was evaluated against T-24 (irrelevant target cells) that do not express GD_2. Only a slight binding of immunotoxin to these non-target cells was observed at 5 – 10 μg/ml concentration. Positive binding of the immunotoxin was also observed with another melanoma cell line (AAB-527, data not shown), indicating the retention of antibody activity after chemical coupling with gelonin.

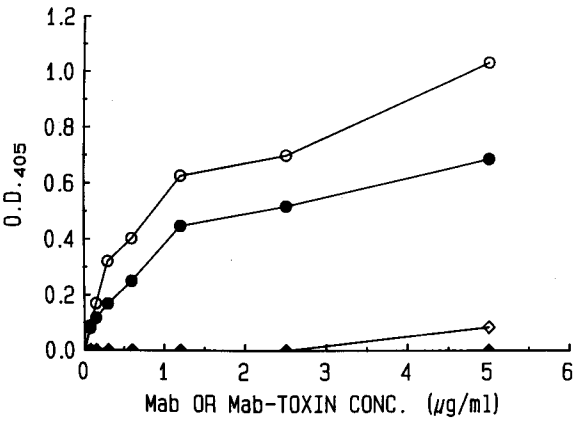

Fig. 2. Immunoreactivity of mAb 14G2a (●——●), 14G2a-gelonin immunotoxin (○——○) on GD_2-positive A375-M melanoma, mAb 14G2a (◆——◆) and (◇——◇) 14G2a-gelonin immunotoxin on GD_2-negative T-24 bladder carcinoma cells

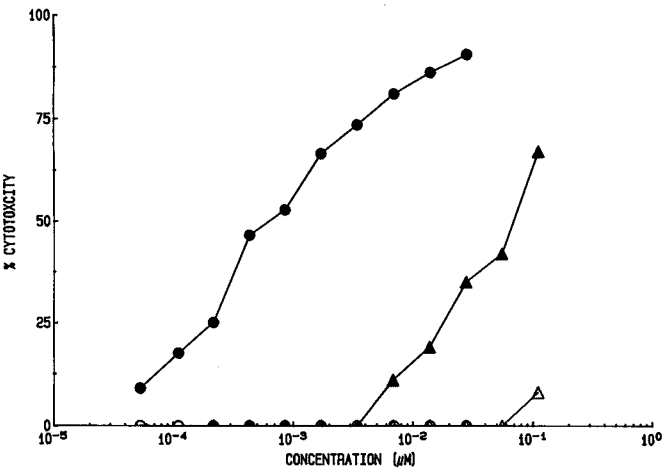

Fig. 3. Antiproliferative effects of 14G2a-gelonin immunotoxin on antigen-positive A375-M (●——●) and AAB-527 (▲——▲) human melanoma cells and antigen-negative (○——○), (△——△) T-24 bladder carcinoma cells when used as a negative control against A375M or AAB-527 melanoma cells respectively. Various concentrations of immunotoxin were added to log-phase cells and incubated for 72 h at 37° C

Cytotoxic activity of 14G2a-gelonin immunotoxin

The in vitro cytotoxic effects of mAb 14G2a and the 14G2a-gelonin immunotoxin against antigen-positive (A375-M, AAB-527) melanoma cells and antigen-negative (T-24) bladder carcinoma cells are shown in Fig. 3. The 14G2a-gelonin immunotoxin was cytotoxic to only antigen-positive A375-M melanoma target cells with an IC_{50} of 0.89 nM. No cytotoxicity was observed with antigen-negative T-24 cells. A 387-fold greater concentration of immunotoxin (344 nM) had to be used to produce a cytotoxic effect on antigen-negative cells. mAb 14G2a alone

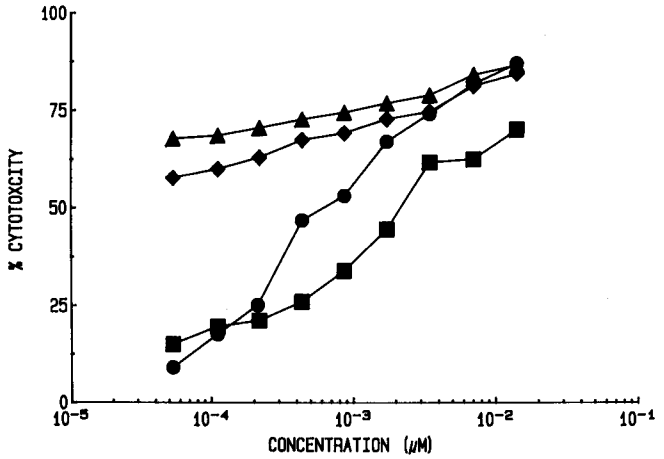

Fig. 4. Effect of various lysosomotropic agents [ammonium chloride (■——■), chloroquine (◆——◆), monensin (▲——▲), 14G2a-gelonin alone (●——●)] on the cytotoxicity of 14G2a-gelonin immunotoxin. Various concentrations of immunotoxin and fixed concentrations of lysosomotropic agents were added to log-phase cells and incubated for 72 h at 37° C

was not cytotoxic to either target or non-target cells (data not shown). The 14G2a-gelonin immunotoxin also showed specific cytotoxicity (IC_{50} = 47 nM) against another melanoma cell line, AAB-527 (Fig. 3). These melanoma cells required a 53-fold greater concentration of immunotoxin than A375-M cells, possibly, in part, because of reduced expression of GD_2 antigen on their surface.

^{125}I-labeled mAb 14G2a was found to be 60%–70% immunoreactive; whereas ^{125}I-labeled 14G2a-gelonin immunotoxin exhibited 40%–50% immunoreactivity. The association constant of mAb 14G2a for A375-M cells was 2.8×10^7 M^{-1} and was 1.0×10^7 M^{-1} for AAB-527. The association constant (K_a) measured for 14G2a-gelonin conjugate was $1.8 \times 10^7 M^{-1}$ for A375M and $0.76 \times 10^7 M^{-1}$ for AAB-527. The average number of binding sites per cell for A375-M was 1.8×10^6 whereas the average number of binding sites/cell for AAB-527 melanoma cells was 3.7×10^5. There was a 4.8-fold difference in the number of binding site between A375-M and AAB-527 melanoma cells. This may partially account for the difference in the cytotoxicity between the two cell lines. However, the difference in the cytotoxicity between the two cell lines could also be caused by different rates of internalization of immunotoxin on A375-M and AAB-527 cells. When the cytotoxicity of 14G2a-gelonin immunotoxin was compared to that of native gelonin with A375-M and AAB-527 melanoma target cells, the immunotoxin was 10^2–10^3 times more cytotoxic than gelonin alone (data not shown).

Effect of various lysosomotropic compounds, biological response modifiers and chemotherapeutic agents on Cytotoxicity of 14G2a-gelonin immunotoxin

Various agents were tested for their ability to increase the intralysosomal pH and are known for their ability to augment immunotoxin-mediated cytotoxicity. Of the lysosomotropic compounds tested 100 nM monensin was most effective in potentiating the cytotoxicity of the 14G2a-gelonin immunotoxin. Monensin (100 nM) treatment resulted in marked augmentation of cytotoxicity (68%) at a 0.053 nM concentration of 14G2a-gelonin immunotoxin. This is in contrast to the results obtained with the immunotoxin alone, in which an IC_{50} of 0.89 nM was observed. Chloroquine (40 μM) also augmented the cytotoxicity of the immunotoxin; approximately 56% cytotoxicity was observed at 0.053 nM concentration of immunotoxin.

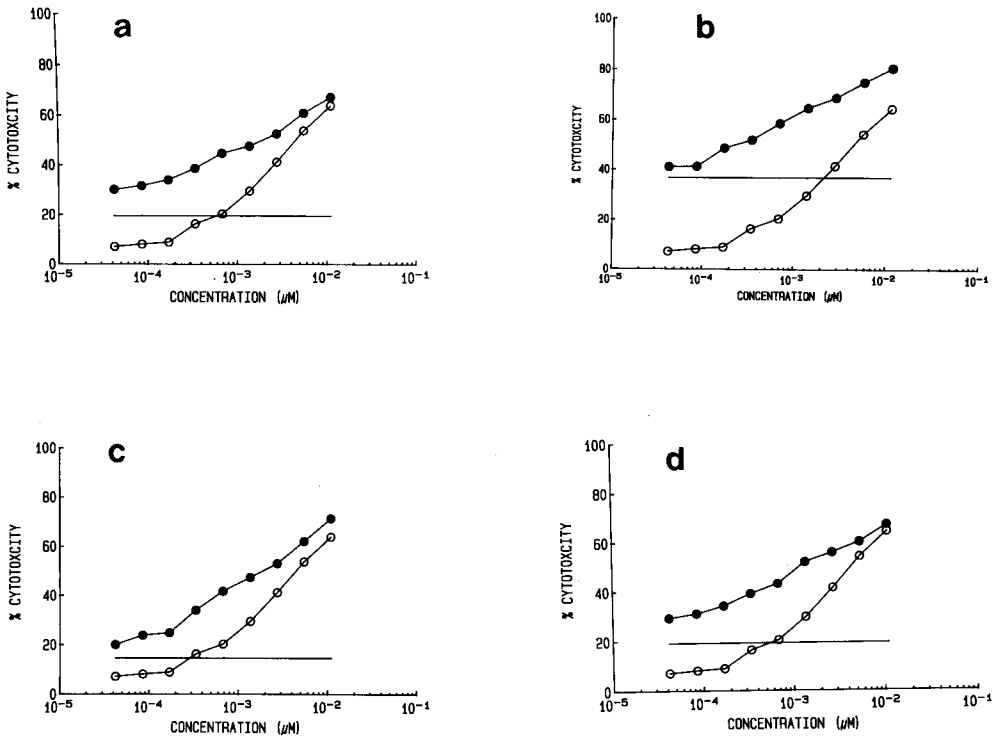

Fig. 5 a–d. Effect of various biological response modifiers and chemotherapeutic agents on the cytotoxicity of a 14G2a-gelonin immunotoxin. Tumor necrosis factor α (TNFα) combined with various concentrations of immunotoxin. TNFα alone exhibited 20% cytotoxicity. **b** The combination of interferonα (IFNα) and 14G2a-gelonin immunotoxin. IFNα alone showed 35% cytotoxicity. **c, d** The augmentation of 14G2a-gelonin cytotoxicity with chemotherapeutic drugs *cis*-platinum (**c**) and carmustine (BCNU) (**d**) respectively. Both cisplatinum and BCNU alone inhibited approximately 12% and 20% of tumor cell growth respectively. ○——○, (In all the graphs) 14G2a-gelonin alone; ●——●, combination of immunotoxin and TNFα (**a**), IFNα (**b**), cisplatinum (**c**) and BCNU (**d**). —, The treatment of cells with biological response modifiers or drugs alone

Table 1. Complement-dependent cytotoxicity with mAb 14G2a and 14G2a-gelonin immunotoxin[a]

[Ab] or [Ab-gelonin] (μg/ml)	Cytotoxicity[b] (%)			
	14G2a	14G2a-gelonin	15A8	15A8-gelonin
20	90±6.0	90.5±2.8	0	0
10	91±3.6	92.5±3.6	0	0
1	75±2.3	40.0±2.4	–	–

[a] NMB-7 (neuroblastoma cells) were labeled with ^{51}Cr and subsequently incubated with mAbs or immunotoxins. Human serum was used as source of complement. mAb 15A8 and 15A8-gelonin conjugate were used as antigen-negative antibody and immunotoxin in this assay. Values shown represent means of two experiments performed in triplicate

[b] Percentage specific lysis

Table 2. Antibody-dependent cellular cytotoxicity with mAb 14G2a and 14G2a-gelonin immunotoxin[a]

[Ab] or [Ab-toxin] (μg/ml)	Target: effector Ratio	Cytotoxicity[b] (%)			
		14G2a	14G2a-gelonin	15A8	15A8-gelonin
10	1:200	58±4.6	41±2.6	0	0
	1:100	46±2.9	34±0.33	0	0
	1: 50	30±0.6	24±1.0	0	0
1	1:200	58±2.1	41±4.6	–	–
	1:100	37±6.0	28±3.0	–	–
	1: 50	26±0.8	16±1.7	–	–
0.1	1:200	39±5.0	25±1.6	–	–
	1:100	30±2.7	22±2.3	–	–
	1: 50	17±0.85	18±0.6	–	–

[a] NMB-7 (neuroblastoma cells) were labeled with ^{51} Cr and incubated with various concentrations of mAbs or immunotoxins, and human peripheral blood mononuclear cells were used as effector cells in this 4-h ^{51}Cr-release assay. Mab15A8 [anti-(carcinoma)] and 15A8-gelonin conjugate were used as irrelevant antibody and conjugate in this assay. Values represent means of two experiments performed in triplicate

[b] Percentage specific lysis

Thus, both chloroquine and monensin were able to augment the cytotoxicity of 14G2a-gelonin immunotoxin by more than one order of magnitude; whereas no potentiation was achieved with ammonium chloride (Fig. 4).

Among the biological response modifiers, TNFα (10 U/ml) showed an additive cytotoxic effect when used in combination with 14G2a-gelonin immunotoxin. The combination of TNFα and immunotoxin resulted in a 5-fold increase in cytotoxicity over that observed with 14G2a-gelonin alone. At a concentration of 10 U/ml TNFα alone caused 20% cytotoxicity (Fig. 5a). Figure 5b demonstrates a 1000-fold augmentation in cytotoxicity when immunotoxin was combined with IFNα. However, at a concentration of 10000 U/ml IFNα alone induced cytotoxicity at the 35% level. No augmentation was seen with 14G2a-gelonin immunotoxin and IFNγ (data not shown). Of a variety of chemotherapeutic agents tested, only cisplatinum (0.05 μg/ml), which binds to DNA, unwinds and disrupts its double helix, and BCNU (15 μg/ml), which inhibits the synthesis of DNA and RNA, showed an additive cytotoxic effect. A 4- to 5-fold augmentation of immunotoxin cytotoxicity was observed when 14G2a-gelonin immunotoxin was combined with cisplatinum (Fig. 5c) and BCNU (Fig. 5d). Cisplatinum (0.05 μg/ml) and BCNU (15 μg/ml) alone caused 12% and 20% inhibition of tumor cell growth respectively. No cytotoxicity either with immunotoxin alone (Fig. 3) or in combination with drugs or cytokines was observed on GD_2-negative T-24 bladder cells (data not shown).

Complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity of 14G2a-gelonin immunotoxin

mAb 14G2a was previously shown [16] to mediate complement-dependent cytotoxicity against melanoma and neuroblastoma target cells with human serum as a source of complement. mAb 14G2a was also shown to mediate ADCC with human peripheral blood mononuclear cells (PBMC) as effector cells against the same target cells. In order to examine whether mAb 14G2a is able to retain these two functional properties as an immunotoxin, CDC and ADCC analyses were made with 14G2a-gelonin immunotoxins. The results depicted in Table 1 indicate that both native antibody and immunotoxin mediate 85%–90% CDC at an antibody and immunotoxin concentration of 1–20 μg/ml. However, once the antibody or antibody-toxin concentration was reduced to 1 μg/ml, mAb 14G2a was still able to mediate 75% CDC compared to only 40% achieved by the immunotoxin. The data shown in Table 2 demonstrate that both mAb 14G2a and 14G2a-gelonin can mediate ADCC with human PBMCs as effector cells. At an antibody concentration of 0.1–10 μg/ml, mAb 14G2a mediates 39%–56% of ADCC at an effector-to-target ratio of 1:200, whereas immunotoxin at the same concentrations mediates 39%–41% of ADCC, demonstrating a slight loss of the functional property of mAb 14G2a once it is coupled to gelonin, which one observes occasionally, caused by the chemical coupling of mAb 14G2a to plant toxin gelonin.

Discussion

mAbs directed against cell-surface antigens or receptors have been widely used as carriers of drugs and toxins [8]. In this report, we describe the coupling of mAb 14G2a to the plant toxin gelonin and evaluation of the immunotoxin (14G2a-gelonin) in vitro against various human melanoma cell lines. mAb 14G2a is directed against disialoganglioside GD_2 expressed on the cell surface and tumor tissues of neuroectodermal origin. This antibody was previously shown [17] to mediate ADCC and CDC effectively using melanoma and neuroblastoma cell lines and has prove to be effective in suppressing the growth of human neuroblastoma xenografts in vivo [17]. Recently, mAb 14G2a also was shown to be effective in achieving partial and complete remissions of tumor growth in neuroblastoma patients (A. Yu and R. Handegretinger, personal communication).

To determine whether mAb 14G2a was able to retain its functional properties after coupling to the plant toxin gelonin, the ability to mediate complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity was tested. The results obtained clearly indicated that the immunotoxin can mediate both CDC and ADCC, as well as the native antibody. These functional properties that were retained by the immunotoxin may be advantageous when one considers this immunotoxin for preclinical and clinical studies. 14G2a-gelonin immunotoxin was immunoreactive and specific when evaluated on ELISA against antigen-positive A375-M and AAB-527 melanoma cells and antigen-negative T-24 bladder cancer cells.

The efficiency of cellular internalization of immunotoxins has been reported to be a critical factor in determining the extent of cellular cytotoxicity. We examined the cytotoxic effects of our immunoconjugate 14G2a-gelonin against two melanoma cell lines expressing GD_2 on their surface. A375-M, which has an IC_{50} of 0.89 nM, and AAB-527 with an IC_{50} of 47 nM. The reason for the 53-fold difference between the IC_{50} of these two melanoma cell lines is partly due to the reduced expression of GD_2 on the surface of AAB-527 melanoma cells. There is a 4.8-fold difference in the numbers of antigen-binding sites between A375-M and AAB-527 melanoma cells. However, Wargalla and Reisfeld [38] have demonstrated that the cellular internalization rate of 14G2a – ricin-A-chain immunotoxin appears to be key to its cytotoxic effects. Therefore, the difference in IC_{50} of 14G2a-gelonin toxin on A375-M and AAB-527 melanoma cells in the current study could also be due to internalization rates between the two cell lines, which is currently under investigation.

The ribosome-inactivating plant toxin gelonin is unable to bind to the cells and therefore is non-toxic except at very high concentrations [33]. However, once coupled to antibody, the immunotoxins generated are highly specific and active in vitro and in vivo [12, 31, 32]. When 14G2a-gelonin immunotoxin was examined against two GD_2-positive human melanoma cells (A375-M and AAB-527) and against GD_2-negative T-24 bladder carcinoma cell lines, it was specifically cytotoxic to the melanoma target cells. This cytotoxicity was due to the binding of mAb 14G2a to target antigen GD_2, since a 200-fold molar excess of mAb 14G2a blocked this cytotoxicity. The immunoconjugate was also 10^2-10^3 times more cytotoxic than native gelonin when tested against both A375-M and AAB-527 melanoma cell lines (data not shown).

Receptor-mediated endocytosis of an antigen-immunotoxin is considered an essential first step in the generation of immunotoxin cytotoxicity. This has been confirmed by using lysosomotropic agents that increase the intracellular pH of acidic vesicles [21] and potentiate the cytotoxicity of immunotoxins [2, 20].

When we examined the effects of various lysosomotropic drugs, we were unable to potentiate the cytotoxicity of 14G2a-gelonin immunotoxin with ammonium chloride. Other investigators [25, 36] have also found only moderate potentiation of immunotoxin cytotoxicity using ammonium chloride. On the other hand, chloroquine augmented the cytotoxicity of the 14G2a-gelonin more than 10-fold. The monocarboxylic ionophore monensin proved to be the most effective in augmenting this cytotoxicity. This finding is consistent with earlier reports by Ramakrishnan et al. [25] and Raso et al. [28].

In addition to monensin and chloroquine, the biological response modifiers TNFα and IFNα augmented the cytotoxicity of the 14G2a-gelonin immunotoxin to some extent. It has been shown that IFNα, and IFNγ can up-regulate the expression of certain melanoma-associated antigens such as P97 [18] and Me14-D12 [9] but not the 240-kDa proteoglycan [6, 11, 18, 19]. In our studies, we were unable to demonstrate any augmentation of 14G2a-gelonin cytotoxicity with IFNγ.

Among the chemotherapeutic agents tested only cisplatinum and BCNU augmented the cytotoxicity of the 14G2a-gelonin immunotoxin 5-fold. Our studies are in agreement with a number of other reports demonstrating the additive and synergistic effects of certain agents such as daunorubicin and IFNγ [39] and monensin [15] with immunotoxins in vivo. The mechanism by which various chemotherapeutic agents augment the cytotoxicity of certain immunotoxins is not known, and the augmentation of cytotoxicity with certain drugs does not seem to be a universal phenomena. In other words, certain drugs that augment the cytotoxicity of one immunotoxin do not augment the cytotoxicity of another immunotoxin. Additional studies are required to understand this phenomenon completely.

We are currently involved in in vivo therapeutics of 14G2a-gelonin immunotoxin compared to antibody or gelonin alone. Should this conjugate have significant in vivo therapeutic effects against human tumors xenografted in mice, these studies may lead to clinical tools for therapy of human melanoma and other tumors of neuroectodermal origin.

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