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Characterization of murine and humanized anti-CD33, gelonin immunotoxins reactive against myeloid leukemias

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Abstract. M195 antibodies recognize CD33, an antigen present on acute myeloid leukemia blasts as well as some myeloid progenitor cells, but not on the ultimate hematopoietic progenitor stem cell. Immunotoxins (IT) reactive with human myeloid leukemias were constructed by conjugating gelonin, a single-chain ribosome-inactivating protein, to murine and genetically engineered, humanized M195 antibodies via an *N*-succinimidyl-3-(2-pyridyldithio)-propionate linkage. No losses of gelonin cytotoxic activity or M195 binding activity were observed after conjugation of up to two toxin molecules per antibody. Toxin conjugates displayed specific, potent toxicity for $CD33^+$ cells. The murine and humanized IT were not toxic to $CD33^-$ cells and were 600 and 4500 times more potent, respectively, than free gelonin in inhibiting $CD33^+$ HL60 cells. Treatment of HL60 cells with 1 μg/ml HuM195-gelonin resulted in more than 1000 times lower colony formation; normal bone marrow mononuclear cell colony-forming units treated with HuM195-IT were reduced by a factor of 10. HL60 leukemia cells could be effectively purged from an excess of normal bone marrow cells. Exposure of target cells to IT for as little as 30 min was as effective as continuous exposure of IT for up to 6 days. However, measures of the efficacy of the immunotoxin were directly related to the length of time of observation after IT exposure and were inversely related to cell concentration. M195-gelonin immunoconjugates are potential candidates for therapeutic use in in vivo or ex vivo bone marrow purging of myeloid leukemias.

Key words: Myeloid leukemia – CD33 – Immunotoxin – Gelonin – M195 – HuM195

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Introduction

Immunotoxins (IT) are a class of proteins that consist of a monoclonal antibody (mAb) covalently linked or genetically fused to a toxic molecule and are thus able to direct potent cytotoxicity to particular cells [41, 22]. Obstacles to effective therapeutic use of immunotoxins for cancer include (a) lack of suitable tumor-specific targets that are not also found on other vital non-tumor cells [39]; (b) loss of toxin potency or mAb activity after conjugation [15]; (c) unwanted cytotoxicity to nontarget cells and tissues resulting from nonspecific internalization of the IT [22]; (d) immunogenicity of the IT [10, 21, 27]; (e) pharmacological inability to target tumor sites adequately [10, 20].

CD33 provides a useful target antigen for therapy of myelogenous leukemias, as it is expressed on the cell surface of more than 80% of leukemia isolates from patients with myeloid leukemia with an average antigen density of 10000 sites/cell [1, 18, 32, 38]. In addition, rapid internalization occurs upon binding of mAb to CD33 both in vitro and in vivo [14, 33]. CD33 is also found on normal granulocyte/monocyte-colony-forming units (CFU-GM), some burst-forming colonies and a fraction of the more primitive progenitors [1, 18, 32, 38]. However, CD33 is not found on tissues outside of the hematopoietic system nor on the normal pluripotent hematopoietic progenitor stem cell. My9, a murine IgG2B mAb reactive with CD33, has been conjugated to a chemically modified ricin toxin and demonstrates potent specific cytotoxicity in vitro [23, 31]. Both M195, a murine monoclonal IgG2a antibody, and HuM195, a humanized version of M195 constructed by genetically grafting the murine CDR regions to a human IgG1 framework and constant regions, are reactive with CD33 [8, 12, 33, 38]. The humanized antibodies may be advantageous because of their reduced immunogenicity, higher avidity, and longer serum half lives [7, 8, 12].

Both murine and humanized M195 are now in clinical trials for the treatment of myelogenous leukemias. ^{131}I-radiolabeled M195 is capable of killing up to 10^{12} leukemia cells in patients with refractory or relapsed leukemias [35]. However, because of the long-range cytotoxicity of the

conjugated nuclide (approximately 50 cell diameters), killing of normal bystander cells occurs as well, requiring bone marrow transplantation at high doses. Use of an anti-CD33 IT may avoid this problem by killing only those cells bearing the CD33 antigen.

Gelonin is a 30-kDa single chain ribosome-inactivating protein isolated from the seeds of *Gelonium multiflorum*, which irreversibly inactivates the ribosomal 60S subunit by cleaving the *N*-glycoside bond of adenine in a specific sequence of rRNA and thereby prevents protein elongation [2, 37]. Gelonin has several advantages over other ribosome-inactivating proteins currently being investigated. Unlike the dual-chain type, such as ricin [4] and abrin [36], which contain a B chain reactive with cell-surface carbohydrates, gelonin is a biochemically stable single chain [22, 29, 36]. It lacks a galactose-specific lectin domain, which is responsible for non-specific binding and toxicity to cells. As a result, free gelonin is much less toxic to intact mammalian cells in vitro and in vivo than the heterodimeric toxins. Despite this relative safety, in a cell-free rabbit reticulocyte translation assay, gelonin demonstrates nearly equal activity to heterodimeric toxins [6, 26]. Attempts have been made to prevent the non-specific binding of the B chain of heterodimeric toxins by separating the A and B chains or by blocking of galactose-binding sites [21, 31]. These methods are technically difficult, variably effective, or sometimes incomplete; hence the IT may contain trace contamination with active B chain that can result in a less selective immunoconjugate and potential toxicity in vivo. Residual membrane-binding activity of the ricin B chain may be necessary for translocation of the A chain [17]. In addition, the larger heterodimers may also be more immunogenic than the single-chain toxins.

Thus, gelonin presents a potential advantage over the dual-chain toxins and was selected for this study. In this paper, we describe immunotoxins constructed by chemical conjugation of mAb reactive against CD33 antigen with the plant toxin gelonin.

Materials and methods

Cell culture. Cell lines HL60 (acute myeloid leukemia, CD33+), U937 (monocytic leukemia, CD33+), Raji and Daudi (B lineage Burkitt's lymphomas, CD33−), Molt4 (T lineage lymphoma, CD33−) and SKLY16 (B lineage lymphoma, CD33−), were maintained in culture using RPMI-1640 medium supplemented with 10% Serum Plus (JRH Biosciences), 5% heat-inactivated newborn bovine serum (Armour Pharmaceuticals), non-essential amino acids, penicillin and streptomycin.

Transformed murine fibroblast cell lines NIH-3T3 and AL67 [24], the latter expressing the transfected CD33 gene, were maintained in culture similarly.

Antibodies. mab M195 and HuM195 were prepared as described [8, 32, 38]. Highly purified HuM195 and HuG1 Fd79 [11], a genetically engineered human IgG1 construct reactive with a herpes simplex virus antigen not found on HL60 cells, were the generous gifts of Man Sung Co, Protein Design Labs, Mountain View, Calif.

SPDP conjugation. A threefold molar excess of *N*-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP), prepared in dry dimethyl-formamide, was added to 10 mg M195 or HuM195 in phosphate-buffered saline pH 7.4 (PBS) and incubated for 30 min at room temperature. Excess SPDP was removed on a Sephadex G-25 column equilibrated with 0.1 M phosphate buffer pH 7.0 containing 0.5 mM EDTA.

Modification of gelonin with 2-iminothiolane. 2-Iminothiolane in 0.5 M triethanolamine hydrochloride (TEA/HCl), pH 8.0, was added to 10 mg gelonin in 60 mM TEA/HCl, 1 mM EDTA, pH 8.0, and incubated for 90 min at 4 °C under nitrogen. Excess 2-iminothiolane was removed by gel filtration on a Sephadex G-25 column equilibrated with 5 mM BISTRIS/acetate buffer pH 5.8 containing 50 mM NaCl and 1 mM EDTA.

Conjugation of M195 with gelonin

SPDP-modified M195 in 100 mM sodium phosphate buffer, 0.5 mM EDTA, pH 7.0, was mixed with a five-molar excess of 2-iminothiolane-modified gelonin. The pH was adjusted to 7.0 with 0.5 M TEA/HCl, pH 8.0, and the mixture was incubated for 20 h at 4 °C under nitrogen. To stop the reaction, iodoacetamide was added to a final concentration of 2 mM and incubated for 1 h at room temperature.

Purification. The reaction mixture was filtered on a Sephacryl S-300 gel filtration column equilibrated with 20 mM TRIS, 50 mM NaCl, pH 7.4, to separate the antibody and antibody-gelonin conjugates from the free gelonin. The fractions containing immunotoxin and unreacted antibody were pooled and then loaded on a Cibacron-blue–Sepharose CL-6B column equilibrated with TRIS buffer to remove the unconjugated antibody. Purified immunotoxin (M195-IT) was eluted with 20 mM TRIS buffer containing 2 M NaCl, pH 7.4. Collected fractions were dialyzed against PBS.

Flow-cytometry assays. Cells were washed and resuspended in RPMI-1640 medium, with 10% Serum Plus, non-essential amino acids, penicillin, streptomycin, and 2% rabbit serum (Pel Freeze). Samples containing 500 000 cells in a final volume of 0.15 ml were incubated for 1 h on ice in the presence of primary antibody. Cells were washed twice, incubated for 1 h on ice with secondary fluorescein-isothiocyanate(FITC)-labelled antibody [goat anti-(mouse Ig) or goat anti-(human Ig)], washed twice, and fixed with 0.5% paraformaldehyde. The FITC fluorescence intensity was measured on an EPICS Profile II flow cytometer [32].

Enzyme-linked immunosorbent assay (ELISA). Primary antibody was added to 96-well plates containing adherent AL67 or NIH-3T3 cells and allowed to bind for 1 h at room temperature. CD33 is overexpressed and is not modulated quickly off of the surface of AL67 cells. Excess antibody was removed by washing the cells three times. Alkaline-phosphatase-labelled secondary antibody was added and allowed to incubate for 1 h at room temperature. Cells were washed three times and 100 µl substrate solution *p*-nitrophenyl disodium phosphate was added. Absorbance at 405 nm was measured using a Fisher Biotek microplate reader after a 10- to 20-min incubation at 37 °C.

Rabbit reticulocyte lysate translation assay. The functional activity of gelonin and gelonin-containing immunotoxin was assayed by a cell-free translation inhibition assay kit (Gibco-BRL) as described by the manufacturer.

Inhibition of [^{3}H]thymidine, [^{3}H]leucine, and ^{3}H-labelled amino acid incorporation. Samples containing 100 µl cells were washed and incubated at 37 °C in 96-well plates in the presence of 50 µl antibody, conjugate, or toxin. After an incubation time of 3–7 days, 50 µl 10 µCi/ml tritiated thymidine, leucine, or amino acids was added to each well and allowed to incorporate for 5–6 h. A 50-µl aliquot of trichloroacetic acid was added to precipitate protein for [^{3}H]-leucine and ^{3}H-labelled amino acid incorporation experiments. Cells were harvested with a Skatron semiautomatic harvester and assayed in a Packard scintillation counter.

3-[4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (thiazoyl blue; MTT) assays. Samples containing 100 µl cells were washed and incubated at 37 °C in 96-well plates in the presence of 50 µl antibody, conjugate, or toxin. After an incubation time of 3–7 days, the plate was centrifuged for 5 min at 1000 rpm. MTT, diluted in PBS, was added to each well and incubated for 4 h. Plates were washed. The formazan product was solubilized with 0.04 M HCl in propan-2-ol and quantified spectrophotometrically at 570 nm.

Competition assays. HL60 cells at 1×10^5 cells/ml were incubated for 1 h on ice in the presence of excess HuM195 or HuFd79. HuM195-IT, at a concentration capable of killing approximately 70% of cells, was then added. Cells were incubated for 90 h at 37 °C then assayed by trypan blue exclusion or [^{3}H]thymidine incorporation.

Time course studies. HL60 cells at 0.67×10^5 cells/ml were incubated with HuM195-IT. At various times cells wee washed twice and re-suspended in an IT-free medium. On day 6 the cells were plated onto a 96-well plate and analyzed by trypan blue exclusion or [^{3}H]thymidine incorporation.

Clonogenic growth. HL60 cells were treated with HuM195-IT and allowed to incubate for 24 h at 37 °C. Cells were then washed and plated in 1 ml 0.3% agarose, RPMI-1640 medium, 8.33% newborn bovine serum, 18.33% fetal calf serum (FCS), non-essential amino acids, penicillin and streptomycin. Plates were incubated for 14 days at 37 °C.

Fourteen-day CFU-GM. Fourteen-day CFU-GM assays were performed essentially as described [16] using various cell concentrations plated onto 1-ml agarose dishes supplemented with granulocyte-colony-stimulating factor, granulocyte/macrophage-colony-stimulating factor and interleukin-3. Growth factors were the generous gift of Janice Gabrilove, MSKCC. Assays were done in duplicate or quadruplicate and each experiment was repeated three times. The plating efficiency was approximately 0.1%.

Purging of HL60 from excess normal bone marrow. Bone marrow aspirates were obtained from normal donors according to Memorial Sloan Kettering Cancer Center IRB protocols. Mononuclear cells were collected by Ficoll-Paque sedimentation, washed, and gamma-irradiated with 8 Gy. Marrow cells were divided into aliquots in 96-well plates at a final concentration of 1×10^6 cells/ml. HL60 cells at a final concentration of 0.667×10^5 cells/ml and HuM195-IT at various concentrations were added to the plates. After a 6-day incubation at 37 °C, cells were assayed for [^{3}H]thymidine incorporation.

Results

Conjugation and purification

Both M195 and HuM195 were conjugated with gelonin and purified as described in the Materials and methods. As shown in Fig. 1, purified M195 antibody migrates on the sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) as three major protein bands representing the glycosylated and carboxyterminal-modified forms of M195 [13]. The final purified conjugate was also found to contain three major protein bands as shown in lane C. Since, electrophoretic analysis alone could not confirm whether the final purified immunotoxin contained any unconjugated antibody, analysis of the final immunotoxin preparation was done by Western blot analysis using anti-gelonin rabbit polyclonal antisera to confirm the presence of gelonin in each of the major Coomassie-stained bands (data not shown).

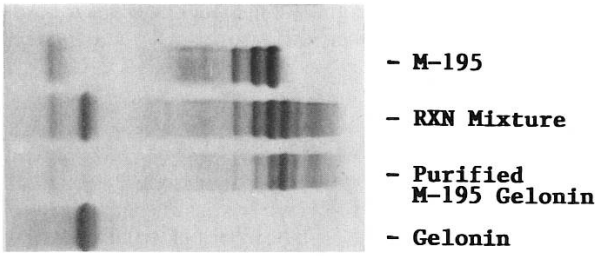

Fig. 1. Sodium dodecyl sulfate gel electrophoretic fractionation of M195 and the immunotoxin M195-IT under non-reducing conditions with a gradient of 5%–20% acrylamide. *Lanes from left to right:* purified mouse M195; reaction mixture containing M195 and gelonin; purified M195-gelonin immunotoxin; purified gelonin

Binding specificity and titer of IT

The binding specificities of M195-IT and HuM195-IT were tested against a variety of CD33$^+$ (U937, HL60, AL67) and CD33$^-$ (SKLY16, Raji, Molt4, Daudi) cell lines using flow cytometry or ELISA. The conjugates retained the same specificity for CD33-expressing cells as the unconjugated antibodies.

Different batches of conjugates containing approximately one or two gelonin molecules per mAb showed no loss of binding titer as compared to unconjugated HuM195 (Fig. 2 A, B) or M195 (data not shown). However, a batch of HuM195 conjugated with an average of three gelonin molecules per mAb, as determined by SDS-PAGE, demonstrated a lower binding titer against both HL60 cells, as assayed by indirect flow cytometry, and AL67 cells, as assayed by ELISA (data not shown). Therefore, all additional experiments presented in this paper used batches of gelonin conjugated to antibody at a ratio of approximately 1 : 1 unless otherwise stated.

Biological activity of toxin

The ability of HuM195-IT and gelonin to inhibit translation in a cell-free system was assayed using a rabbit reticulocyte lysate translation assay. Both the immunotoxin and free gelonin demonstrated a similar 50% inhibitory concentration (ID_{50}) in the range of 12–16 pM, demonstrating that conjugation of gelonin to the antibody did not alter the activity of the toxin (Fig. 3).

Cytotoxicity of immunoconjugates. Both M195-IT and HuM195-IT were tested for their ability to kill CD33$^+$ and CD33$^-$ cells in comparison to free gelonin. Cytotoxicity was determined by a variety of methods including inhibition of incorporation of tritiated amino acids into trichloroacetic-acid-precipitable protein, inhibition of DNA synthesis, trypan blue exclusion, MTT, and clonogenic assays (not all shown). M195-IT had an ID_{50} of approximately 400 pM, which was approximately 600 times more potent than free gelonin (Fig. 4). HuM195-IT, which has a higher affinity for CD33 than M195, had an ID_{50} of 15 pM, which was 4500 times more potent than the ID_{50} of free gelonin (Fig. 5). Different lots of HuM195-IT displayed

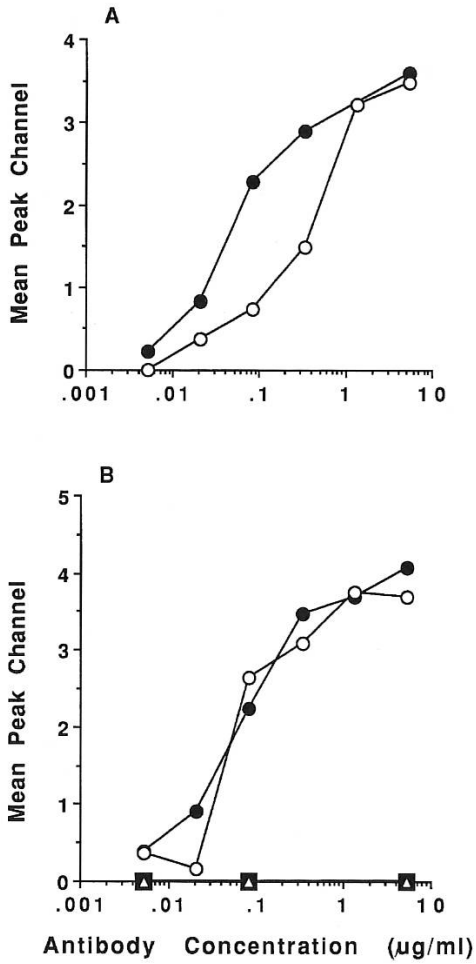

Fig. 2 A, B. Binding titer and specificity of HuM195-IT on cell lines. HL60, U937, or Molt4 cells at a final concentration of 1.5×10^6 cells/ml were incubated on ice for 1 h with either HuG1 M195 or HuG1 M195-IT a a final concentration range of 0.08 μg/ml to 10 μg/ml. Mean peak fluorescence intensity (*y* axis) versus mAb or immunotoxin (IT) concentration (*x* axis) was measured using an EPICS Profile II cytometer. **A** HL60 binding by HuM195-IT (●) or by HuM195 (○). **B** U937 binding by HuM195-IT (●) or by HuM195 (○); Molt4 binding by HuM195-IT (■) or by HuM195 (△)

different levels of cytotoxicity with some lots having an ID_{70} of 5 pM. There was a slow loss of potency over time (months) suggesting either reduction of the intermolecular linkage or denaturation of the IT. Because of the variabilities, accurate comparisons of potency between the murine and humanized conjugates are not possible.

Cytotoxicity, as determined by ^{3}H-labelled amino acid or [^{3}H]thymidine incorporation, was confirmed to result in cell death by examining parallel wells by trypan blue analysis or similarly treated cells as measured by MTT (not shown).

Non-specific cytotoxicity was not observed with the IT. Even at the highest concentration of immunotoxin used, typically 10–16 μg/ml, no cytotoxicity was observed when either conjugate was incubated with the CD33-negative cell lines Raji, Molt4, Daudi, and SKLY16 (not shown).

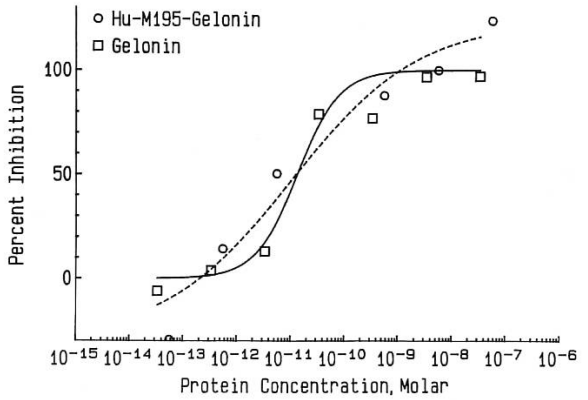

Fig. 3. Rabbit reticulocyte lysate translation assay. Increasing amounts of free gelonin toxin (□) or HuM195-IT (○) were assayed for activity in a cell-free lysate translation inhibition assay. HuM195-IT final concentrations ranged from 5 pg/ml to 5 μg/ml. Gelonin final concentration ranged from 1 pg/ml to 1 μg/ml

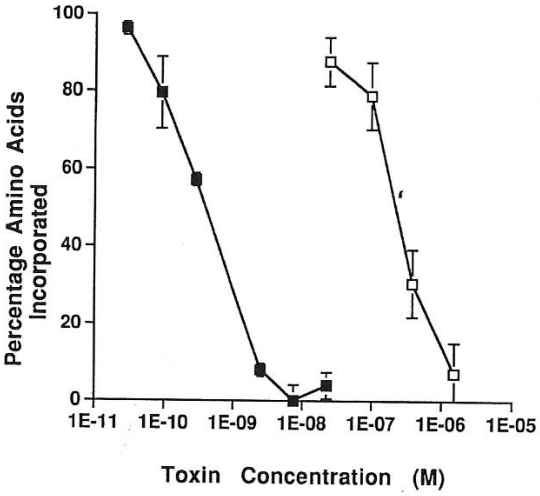

Fig. 4. Inhibition of protein synthesis in live cells by gelonin and M195-IT on HL60 cells. HL60 cells at a final concentration of 1×10^6 cells/ml were incubated for 3 days at 37 °C in the presence of M195-IT (■) and gelonin (□). Levels of protein synthesis were determined by 5 h incorporation of tritiated amino acids into trichoroacetic-acid-precipitable protein. M195-IT final concentrations ranged from 5 ng/ml to 4 μg/ml. Gelonin final concentration ranged from 0.5 μg/ml to 50 μg/ml. The data are representative of four experiments

Because of the increased potency shown above and the theoretical advantage of reduced immunogenicity of the humanized form of the M195 in vivo, all further experiments were conducted using the HuM195-IT.

HuM195-IT inhibited the clonogenic growth of HL60 in a dose-dependent manner. Incubation of cells with 10 μg/ml and 1 μg/ml HuM195-IT for 72 h decreased colony formation from 8225 colonies/10^5 cells plated to 5 and 13 colonies/10^5 cells plated respectively (Fig. 6). Incubation with 0.1 μg/ml HuM195-IT and unconjugated antibody did not significantly inhibit growth.

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