Humanized M195 Monoclonal Antibody Conjugated to Recombinant Gelonin: An Anti-CD33 Immunotoxin with Antileukemic Activity

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ABSTRACT

The recently characterized immunotoxin HuM195-gelonin consists of a humanized anti-CD33 monoclonal antibody conjugated to the single-chain plant toxin gelonin. Binding of the immunotoxin to hematopoietic cells that express the CD33 differentiation antigen has been demonstrated and results in cytotoxicity due to ribosomal inactivation by gelonin. Blast cells from most patients with acute myelogenous leukemia express CD33, whereas normal stem cells necessary for maintenance of hematopoiesis do not. We asked whether an immunoconjugate using recombinant gelonin rather than plant gelonin is toxic to acute myelogenous leukemia (AML) cell lines and primary AML blasts obtained from patients and exposed to the immunotoxin in vitro. The CD33pos cell lines HL60, OCIAML2, and OCI-AML3 showed decreased proliferation when exposed to immunotoxin for 24–72 h. The CD33pos cell line OCI/AML3 was relatively resistant to HuM195, and all cell lines were resistant to equimolar concentrations of unconjugated antibody and gelonin. Primary blast cultures from seven patients with AML had CD33 detectable on 75.7–99.8% of cells by flow cytometry, and all showed dose-dependent decreases in clonogenic cell survival during 24-h incubation with the immunotoxin. Cells selected for low CD33 expression by cell sorting or by prolonged incubation with immunotoxin could reexpress CD33 at baseline levels and remained sensitive to immunotoxin. We conclude that humanized M195 conjugated to recombinant gelonin has antileukemic activity and should be considered for clinical testing in Phase I trials.

INTRODUCTION

The development of cell-targeted cytotoxic agents holds potential for the treatment of hematopoietic malignancies as suggested by clinical responses observed in Phase I trials with a variety of immunoconjugates (1–3). Monoclonal antibodies targeting cell surface antigens and growth factors such as interleukin 2 fused or coupled to toxins have been studied as potential therapeutic agents, but success has been limited by obstacles such as immunogenicity, lack of specific tumor antigens, and adverse effects due to the extreme toxicity of plant and bacterial toxins used (4). The design of new immunotoxins requires the use of strategies that address these problems. CD33 is found on the surface of myeloid cells in normal hematopoiesis and has structural and binding characteristics that identify it as a member of the sialectakin family of proteins (5). The exact function of CD33 is not known but may involve adhesion or cell-cell communication. CD33 is also expressed on blast cells in most cases of AML but is not expressed on the earliest normal hematopoietic stem cells (6).

CD33 is a target at the surface of most leukemic cells in patients with AML that may be useful for the development of targeted therapeutic agents (7, 8). The anti-CD33 monoclonal antibodies M195 (murine) and HuM195 (humanized) are now in clinical trials for the treatment of AML.111I-Radiolabeled M195 localizes to peripheral blood and marrow blast cells in patients with AML (7). M195 is a murine monoclonal IgG2a, whereas HuM195 consists of murine complementarity-determining regions genetically grafted to a human IgG1 framework and constant regions (9). HuM195 has preserved binding affinity for CD33 but is not immunogenic and has a serum half-life of 42 h (8). Conjugation of gelonin with HuM195 did not appear to interfere with binding to CD33. McGraw et al. (10) recently characterized humanized M195-gelonin, which is a conjugate of HuM195 and the plant toxin gelonin, and which is reactive against the CD33pos myeloid leukemia cell line HL60 in vitro (10).

Gelonin toxin was originally isolated from the seeds of *Geloinum multiflorum*. It is a M, 30,000 single-chain protein that inactivates the ribosomal 60S subunit by cleaving RNA adenine N-glycoside bonds in a sequence-specific fashion (11). Unlike dual-chain toxins such as ricin, gelonin lacks a carbohydrate-binding domain and, by itself, cannot bind to cells and

1 The abbreviations used are: AML, acute myelogenous leukemia; rGel, recombinant gelonin; HuM195-rGel, humanized M195 monoclonal antibody conjugated to rGel; FACS, fluorescence-activated cell sorting; CFU, colony-forming unit.
is relatively nontoxic. Conjugated to an internalizing antibody, however, gelonin is highly toxic, inhibiting protein synthesis irreversibly. Recently, we reported the sequencing, cloning, and bacterial expression of a recombinant gelonin protein (rGel) with identical biological properties to the natural toxin (12). The studies reported herein used the recombinant toxin in contrast to previous studies that used natural gelonin.

HuM195-gelonin was found previously to have activity against the myeloid cell line HL60 in vitro (10). We asked whether similar activity could be demonstrated with HuM195-rGel using blast cells obtained freshly from patients with AML and maintained in liquid culture medium. A cloning culture system was chosen to measure the effect on AML blast progenitor cells specifically. We report that HuM195-rGel is active against AML cell lines and primary cultures and that this effect is specific to CD33-positive cells.

MATERIALS AND METHODS

Preparation of Immunotoxin. HuM195 was prepared as described (9) and was generously supplied by Protein Design Labs, Inc. (Mountain View, CA). rGel was expressed in Escherichia coli and purified as described previously (12). Conjugation of HuM195 with rGel was also performed as described previously (10). Briefly, N-succinimidyl 3-(2-pyridylthio)propionate-modified HuM195 in 100 mM sodium phosphate buffer, 0.5 mM EDTA (pH 7.0), was mixed with a 5-molar excess of 2-iminothiolane modified rGel. The pH was adjusted to 7.0 with 0.5 mM triethanolamine hydrochloride (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. The reaction mixture was purified on a Sephacryl S-300 gel filtration column equilibrated with 20 mM Tris and 50 mM NaCl (pH 7.4). 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 fragments. Antibody fragments were obtained from Caltag (San Francisco, CA) and used at a dilution of 1:5.

Cell Lines. HL60, OCI/AML2, OCI/AML3, and OCI/AML5 were maintained in α minimal essential medium supplemented with 10% fetal bovine serum (Life Technologies, Inc., Grand Island, NY), at 37°C with 5% CO₂ in a humidified incubator. Maintenance cultures of OCI/AML5 were also supplemented with 0.1 ng/ml recombinant granulocyte/macrophage colony-stimulating factor, 0.1 ng/ml granulocyte colony-stimulating factor, and 1 ng/ml stem cell factor (R and D Systems).

Clonogenic Cultures. AML cell lines or patient samples were incubated in liquid culture for 24–72 h with HuM195-rGel, rGel, or HuM195 and rGel at the concentrations indicated, with a cell density of one million per ml at the beginning of the experiment. Cells were then washed twice in α minimal essential medium, resuspended in plating solution (15% fetal bovine serum, growth factors, and 0.8% methylcellulose), and plated in quadruplicate. 0.1 ml/well, in 96-well microtiter plates (Linbro, Horsham, PA). The amount of cell suspension added to plating solution corresponded to equal volumes of liquid culture from which cells had been taken. Colonies consisting of >20 cells were counted after 5 to 7 days of incubation at 37°C with 5% CO₂. To determine the effect of cryopreservation, cells were exposed to HuM195-rGel (1 nM) for 24 h, washed, resuspended in medium with 10% DMSO and 50% FBS in 6.0-ml tubes, and placed in a −70°C freezer for a minimum of 24 h. Cells were then quickly thawed by immersion in a 37°C water bath, washed, incubated at 37°C for 24 h, washed again, and plated in methylcellulose. Control cells were incubated for 24 h at the same temperature and cell density as thawed cells, then plated.

Flow Cytometric Analysis and Sorting. Bone marrow aspirates from patients with AML were analyzed for CD33 expression using a standardized protocol in the Flow Cytometry laboratory at the University of Texas M. D. Anderson Cancer Center, Division of Laboratory Medicine. CD33 expression of cell lines was detected using a CD33 antibody labeled with phycoerythrin (Becton Dickinson, San Jose, CA). To detect bound immunotoxin, FITC-labeled goat anti-human-IgG F(ab)₂ antibody fragments were obtained from Caltag (San Francisco, CA) and used at a dilution of 1:5. The analysis was done using a FACSscan (Becton Dickinson, San Jose, CA), gated on live cells by scatter criteria. For all staining procedures, nonspecific binding was controlled for using an irrelevant antibody of the same isotype and was subtracted. Incubations were performed on ice (PBS, 0.2% BSA, and 0.1% NaN₃). FACS sorting was performed under sterile conditions using a FACSVantage (Becton Dickinson, San Jose, CA).

RESULTS

Cytotoxicity of HuM195-rGel Is Specific for Cell Lines That Express CD33. HL60 colony formation was inhibited by HuM195-rGel in a dose-dependent manner (Fig. 1), with a log reduction in clonogenic cells obtained after 72-h incubation with 5 nm immunotoxin. This is consistent with results published previously with HuM195-gelonin (10). Colonies that did form after exposure to HuM195-rGel were smaller than colonies from untreated cells or cells exposed to unconjugated antibody and rGel or free rGel alone, indicating a degree of growth-inhibitory effect on all cells in the culture. The AML cell lines OCI/AML2 and OCI/AML5 are CD33-positive and showed decreased colony formation in response to 48-h incubation with HuM195-rGel but were not growth inhibited by equivalent concentrations of unconjugated HuM195 and rGel. OCI/AML3 does not express CD33, and colony formation was not inhibited by HuM195-rGel (data not shown).
Blast Cells Obtained Freshly from Patients with AML Are Sensitive to HuM195-rGel in Vitro. We determined the effect of HuM195-rGel on AML blasts in primary culture using a combination of liquid cultures and methylcellulose cultures. Samples were obtained from 15 patients with newly diagnosed or relapsed AML and 1 patient with chronic myelogenous leukemia blast crisis. The CD33 expression measured by flow cytometry on bone marrow blasts ranged from 75.7 to 99.8%. Incubation for 24 h in liquid culture with HuM195-rGel resulted in dose-responsive decreases in clonogenic cell recovery for each of seven patient samples as measured by colony formation in methylcellulose-containing medium. Growth inhibition due to equimolar concentrations of unconjugated HuM195 and rGel was seen only at very high concentrations (≥100 nM). The IC50 of immunotoxin was 1–30 nM for patient samples and 0.3 nM for HL60 (24-h incubation). In contrast, the IC50 of unconjugated monoclonal antibody and rGel (mixture of both molecules, each at the stated concentration) was 100–200 nM (Table 1). Prolonging the incubation time to 72 h had a variable effect on IC50 for patient samples but consistently lowered the IC50 for unconjugated antibody and rGel.

Patient samples had varying CD33 expression, measured on bone marrow blasts by flow cytometry, as the percentage of cells with positive staining. Although this method confirms the presence of CD33 on the cell surface, it may not give an accurate measure of receptor density. We asked whether results obtained from flow cytometry would correlate with sensitivity to immunotoxin. Fig. 2 shows the trend of lower IC50 with increasing CD33 positivity (r = –0.25, P = 0.59).

Recent studies in our laboratory4 have demonstrated that doses of HuM195-rGel up to 12.5 mg/kg can be safely administered i.v. to mice. At this dose, blood concentrations of up to 12.5 μg/ml (70 nM) should be safely attained and are well above the IC50 concentrations for blast cells from AML patients.

**Potency of HuM195-rGel Antileukemic Effect.** To further evaluate the potency of HuM195-rGel with respect to log-kill, we determined empirically the IC50 for HL60 and patient 2 (data not shown). The IC50 for HL60 shows a 4-log difference in sensitivity to HuM195-rGel versus free rGel, whereas the IC90 shows a 2-log difference. The patient sample was more sensitive to immunotoxin than to unconjugated antibody and rGel by a factor of 25 at the IC50 and by a factor of 9 at the IC90. These results indicate that the degree of antibody-

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Table 1  IC50 of immunotoxin versus unconjugated monoclonal antibody and toxin for HL60 and seven AML patient samples

<table>
<thead>
<tr>
<th>Patient</th>
<th>IC50 (nM)</th>
<th>IC50 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24-h incubation</td>
<td>72-h incubation</td>
</tr>
<tr>
<td></td>
<td>Immunotoxin</td>
<td>HuM195</td>
</tr>
<tr>
<td>HL60</td>
<td>0.3</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Patient 1</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>Patient 2</td>
<td>4.0</td>
<td>100</td>
</tr>
<tr>
<td>Patient 3</td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td>Patient 4</td>
<td>30</td>
<td>200</td>
</tr>
<tr>
<td>Patient 5</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Patient 6</td>
<td>10</td>
<td></td>
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<td>Patient 7</td>
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</tbody>
</table>

* Fifty % inhibitory concentration estimated from plot of colonies formed versus concentration of immunotoxin or unconjugated equimolar HuM195 and rGel.

* Cells incubated in suspension culture with immunotoxin or unconjugated molecules for time shown and then washed and plated for colony formation.

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4 M. Rosenblum, unpublished data.
mediated specificity was not uniform across the dose-response range, but that there was measurable targeting at concentrations necessary for 1-log reduction in clonogenic cells during a 24-h incubation.

**HuM195-rGel Has Synergy with Cryopreservation.** Treatment of AML with high-dose chemotherapy followed by transplantation of cryopreserved autologous bone marrow is frequently unsuccessful due to the presence of leukemic blast progenitors in the bone marrow autograft. Cryopreservation itself has some antileukemic effect, to which other purging modalities have been added (14). To determine whether cryopreservation would enhance the effect of HuM195-rGel against CD33<sup>pos</sup> AML blasts, we suspended cells from 12 patient samples in freezing medium immediately after exposure to HuM195-rGel (1 nM), then determined clonogenic cell recovery after thawing. Data shown in Fig. 3 indicate that there is a greater than additive effect against all but one patient sample.

**CD33 Is Reexpressed, and Sensitivity to HuM195-rGel Is Maintained after Selection for CD33<sub>low</sub> Cells.** Measurement of CD33 expression by flow cytometry reveals a unimodal distribution within which there are individual cells with lower or higher CD33 expression. We asked whether cells that are relatively resistant to HuM195-rGel on the basis of low CD33 expression would produce more highly expressing cells on clonal expansion or give rise to a resistant clone with stably down-regulated CD33 expression. HL60 cells were separated into CD33-bright and CD33-dim populations by FACS. CD33 expression was measured again by flow cytometry after 6 days in culture. Both populations were 100% CD33<sup>pos</sup> with mean channel numbers of 166.7 and 166.3, respectively.

The persistence of CD33 expression was also reflected in the dose-response curves of OCI/AML5 after preincubation for 0–6 days with immunotoxin at an inhibitory concentration. After 2, 4, or 6 days exposure to immunotoxin, OCI/AML5 continued to show sensitivity to HuM195-rGel that is stable, although not as great as that seen with naive cells (Fig. 4). Cells incubated with immunotoxin for 5 days showed only faint staining for CD33 (median fluorescence intensity, 5.6 fluorescence units versus 196.1 fluorescence units in the control not treated with immunotoxin). To confirm that there was a decrease in the density of immunotoxin binding sites, cells were then exposed to fresh immunotoxin followed by FITC-labeled anti-human IgG. Pretreated cells again showed decreased median fluorescence intensity (85 fluorescence units versus 498 fluorescence units in naive cells), indicating decreased immunotoxin binding. CD33 expression was restored after incubation for 5 days in immunotoxin-free media (205 fluorescence units in pretreated versus 220 fluorescence units in untreated cells). The transient decrease in CD33 positivity correlates with the expected time course for internalization of immunotoxin-bound CD33 molecules and expression of new CD33 on the cell surface (8).
row cultures and SCID-Hu (severe combined immunodeficiency mice with human xenograft mice; Ref. 6). In contrast, AML blast progenitors usually show a high proliferative fraction (50% in S phase) and express growth factor receptors, resembling the more differentiated CD34<sup>pos</sup>/CD33<sup>pos</sup> progenitors in normal hematopoiesis. Raymakers et al. (18) reported the morphology and clonal marker status of colonies grown from CD34<sup>pos</sup>/CD33<sup>pos</sup> versus CD34<sup>pos</sup>/CD33<sup>neg</sup> blasts from 33 patients with AML. CD34<sup>pos</sup>/CD33<sup>neg</sup> cells from patients whose blasts were predominantly CD33<sup>pos</sup> gave rise to multilineage colonies that were diploid (lacking clonal markers in five patients with cytogenetic abnormalities), demonstrating that these were residual normal stem cells rather than CD33<sup>neg</sup> leukemic progenitors. AML blasts with more immature phenotype (French-American-British classification: M1 and M2) tend to have lower CD33 expression and most likely can include CD33<sup>neg</sup> leukemic progenitors in some cases (18).

The antileukemic effect of HuM195-rGel is similar to that reported previously by Roy et al. (19) using a CD33-directed immunotoxin containing modified, intact ricin. In clinical trials, however, immunotoxins incorporating ricin have been associated with generalized vascular changes that are often life-threatening and most likely due to the antibody-independent effects of blocked ricin, which retains significant toxicity toward the vascular endothelium (1, 2, 20). In contrast, free rGel appears to be less toxic; yet our results show that conjugation with an anti-CD33 antibody yields an immunotoxin with selective toxicity to HL60 and primary AML blasts comparable with that reported for anti-MY9-br (MY9 monoclonal antibody conjugated to blocked ricin). Inhibition of normal hematopoietic progenitors in vitro has also been demonstrated with anti-MY9-br and would be expected with HuM195-rGel. Direct comparison of CFU-leukemic with CFU-granulocyte/macrophage, blast forming unit-erythroid, or CFU-granulocyte-erythrocyte-megakaryocyte-macrophage should not be used to predict in vivo therapeutic advantage, however, because the respective progenitors respond differently to cell culture conditions.

Phase I studies in humans are needed to assess the toxicity and immunogenicity of HuM195-rGel. The in vivo effects of HuM195-rGel in nude mice with HL60 xenografting have been reported separately and demonstrate that antileukemic concentrations of the immunotoxin can be achieved in vivo without significant toxicity (21). Patient samples were not as sensitive as HL60 but are nevertheless susceptible to killing by concentrations achievable in vivo. The therapeutic potential of an immunotoxin can be limited if the molecule is immunogenic when administered in vivo. Treatment with murine monoclonal antibodies is typically followed by the appearance of human anti-mouse antibody, which can neutralize an immunotoxin (4, 7). HuM195 contains murine complementarity-determining regions only; the constant and variable framework domains consist of human IgG1 sequences. In a Phase I trial, HuM195 did not induce the formation of human anti-mouse antibody and could be administered repeatedly (8). It remains to be determined whether HuM195-rGel is immunogenic in humans, however, because there may be an immune response to rGel. We conclude that HuM195-rGel is active in vitro against AML, and that this immunotoxin merits further evaluation for potential use in the...

**DISCUSSION**

AML blasts that express CD33 were killed by exposure to HuM195-rGel. With fresh blasts from patients with AML, a 24-h exposure was sufficient to demonstrate dose-dependent decreases in clonogenic cell survival. Further increases in antibody-mediated toxicity were not consistently observed with more prolonged incubation times, although nonspecific toxicity was enhanced. This finding suggests that brief exposure to immunotoxin in vivo is likely to yield the greatest difference in response between targeted and nontargeted cells. Dose-related killing of clonogenic cells was observed at the highest concentrations of immunotoxin tested. These results indicate that AML blast progenitor cells are sensitive to HuM195-rGel because only the progenitor cells are capable of forming colonies (15). The immunonjugate HuM195-rGel is toxic to hematopoietic cells expressing CD33 because binding to the cell surface occurs and is followed by internalization of the molecule by the cell. Only a small number of internalization events are required to transport enough rGel into the cell to irreversibly inhibit protein synthesis, leading to death of the cell (10, 16). Nonhematopoietic tissues do not express CD33 because binding to the cell surface occurs and is followed by internalization of the molecule by the cell. Only a small number of internalization events are required to transport enough rGel into the cell to irreversibly inhibit protein synthesis, leading to death of the cell (10, 16). Nonhematopoietic tissues do not express CD33 because binding to the cell surface occurs and is followed by internalization of the molecule by the cell. Only a small number of internalization events are required to transport enough rGel into the cell to irreversibly inhibit protein synthesis, leading to death of the cell (10, 16). Nonhematopoietic tissues do not express CD33 because binding to the cell surface occurs and is followed by internalization of the molecule by the cell. Only a small number of internalization events are required to transport enough rGel into the cell to irreversibly inhibit protein synthesis, leading to death of the cell (10, 16).

It is likely that some targeted cells survive a single exposure to immunotoxin. An important question arising from our study is whether CD33<sup>pos</sup> or CD33<sup>neg</sup> AML blasts constitute a small population of progenitor cells, for which there is evidence in normal hematopoiesis, which would be resistant to immunotoxin. Wagner et al. (6) divided CD34<sup>pos</sup> human hematopoietic progenitors into groups based on size and found that the fraction of cells that lacked CD33 expression was a phenotypically primitive, quiescent population (97% in G<sub>0</sub>-G<sub>1</sub>), with the highest multilineage proliferative potential in long-term bone marrow cultures and SCID-Hu (severe combined immunodeficiency mice with human xenograft mice; Ref. 6). In contrast, AML blast progenitors usually show a high proliferative fraction (50% in S phase) and express growth factor receptors, resembling the more differentiated CD34<sup>pos</sup>/CD33<sup>pos</sup> progenitors in normal hematopoiesis. Raymakers et al. (18) reported the morphology and clonal marker status of colonies grown from CD34<sup>pos</sup>/CD33<sup>pos</sup> versus CD34<sup>pos</sup>/CD33<sup>neg</sup> blasts from 33 patients with AML. CD34<sup>pos</sup>/CD33<sup>neg</sup> cells from patients whose blasts were predominantly CD33<sup>pos</sup> gave rise to multilineage colonies that were diploid (lacking clonal markers in five patients with cytogenetic abnormalities), demonstrating that these were residual normal stem cells rather than CD33<sup>neg</sup> leukemic progenitors. AML blasts with more immature phenotype (French-American-British classification: M1 and M2) tend to have lower CD33 expression and most likely can include CD33<sup>neg</sup> leukemic progenitors in some cases (18).

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treatment of patients with AML or for ex vivo purging of bone marrow for autologous bone marrow transplantation.

REFERENCES


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