Anti-CD38-Blocked Ricin: An Immunotoxin for the Treatment of Multiple Myeloma

By Victor S. Goldmacher, Lizabeth A. Bourret, Beth A. Levine, Robert A. Rasmussen, Majid Pourshadi, John M. Lambert, and Kenneth C. Anderson

We report the development of a potent anti-CD38 immunotoxin capable of killing human myeloma and lymphoma cell lines. The immunotoxin is composed of an anti-CD38 antibody HB7 conjugated to a chemically modified ricin molecule wherein the binding sites of the B chain have been blocked by covalent attachment of affinity ligands (blocked ricin). Conjugation of blocked ricin to the HB7 antibody has minimal effect on the apparent affinity of the antibody and no effect on the ribosome-inactivating activity of the ricin A-chain moiety. Four to six logs of CD38+ tumor cell line kill was achieved at concentrations of HB7-blocked ricin in the range of 0.1 to 3 nmol/L. Low level of toxicity for normal bone marrow (BM) granulocyte-macrophage colony-forming units (CFU-GM), burst-forming units-erythroid (BFU-E), colony-forming units-granulocyte/erythroid/monocyte/macrophage (CFU-GEMM) cells was observed. Greater than two logs of CD38+ multiple myeloma cells were depleted from a 10-fold excess of normal BM mononuclear cells (BMMCs) after an exposure to HB7-blocked ricin under conditions (0.3 nmol/L) that were not very toxic for the normal BM precursors. HB7-blocked ricin was tested for its ability to inhibit protein synthesis in fresh patients’ multiple myeloma cells and in normal BMMCs isolated from two healthy volunteers; tumor cells from four of five patients were 100-fold to 500-fold more sensitive to the inhibitory effect of HB7-blocked ricin than the normal BM cells. HB7 antibody does not activate normal resting peripheral blood lymphocytes, and HB7-blocked ricin is not cytotoxic toward these cells at concentrations of up to 1 nmol/L. The potent killing of antigen-bearing tumor cells coupled with a lack of effects on peripheral blood T cells or on hematopoietic progenitor cells suggests that HB7-blocked ricin may have clinical utility for the in vivo or in vitro purging of human multiple myeloma cells.

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MULTIPLE MYELOMA remains an incurable malignancy with a median survival of only 48 months when conventional therapies are used. Whether combination chemotherapy is superior to melphalan and prednisone remains controversial. The recent use of α2b interferon during initial treatment and/or as a maintenance therapy, and the use of high doses of chemoradiotherapy followed by hematopoietic stem cell transplantation have both shown promise in some preliminary studies for selected patients with myeloma. However, at present, myeloma remains incurable, and innovative treatment strategies are needed.

Immunotoxins or hormonotoxins formed by conjugation of potent toxins to monoclonal antibodies (MoAbs) or hormones that bind to cell surface molecules on myeloma cells represent a potentially useful novel treatment strategy. For example, based upon the necessary role of interleukin-6 (IL-6) in the growth of myeloma cells and the presence of IL-6 receptors on the surface of such cells, IL-6 has been linked to pseudomonas exotoxin and diphtheria toxin with the hope of using the IL-6 receptor pathway for the delivery of these toxins specifically to myeloma cells. The widespread expression of IL-6 receptors on normal tissues, including hematopoietic stem cells, may limit the potential clinical utility of such toxins. Other antigens on the cell surface of myeloma cells include those present on normal B cells at various stages of differentiation from the pre-B to the plasma cell as well as antigens expressed on non-B cells. To date, an immunotoxin to target myeloma cells has been prepared by conjugating the 8A MoAb, which recognizes both plasma cells and B-cell precursors to the ribosome-inactivating protein momordin.

The most strongly and uniformly expressed antigen on cells identified on clonal populations of myeloma cells is CD38. This antigen has been recently identified as nicotinamide adenine dinucleotide (NAD) glycohydrolase and is expressed in all myelomas, most cases of T- and B-lineage acute lymphoblastic leukemias, some acute myelocytic leukemias, follicular centre cell lymphomas, and T-lymphoblastic lymphomas. Only a few normal cell lineages express CD38: B cells, including pre-B cells, B cells, and plasma cells; T cells, including cortical thymocytes and activated T cells; basophils; and a subpopulation of macrophages. The strong expression of CD38 on myeloma patients’ malignant plasma cells compared with its pattern of expression on normal cells suggests that this antigen may be a useful target for the in vivo or in vitro depletion of tumor cells while sparing normal cells. Indeed, a chimeric heteroconjugate using the Fab portion of an anti-CD38 antibody and a human IgG Fc fragment has been shown to mediate antibody-dependent cellular cytotoxicity and is undergoing clinical testing.

In this report, we describe the in vitro cytotoxic properties of a conjugate of the anti-CD38 MoAb HB7 with ricin that has been chemically modified so that its galactose-binding sites are blocked by covalently attached affinity ligands. This HB7-blocked ricin conjugate shows a 4- to 6-log depletion of CD38+ malignant plasma cells at concentrations that do not significantly alter the in vitro proliferation of hematopoietic progenitor cells. The potent specific cytotoxicity of this immunotoxin for tumor cells combined with its low cytotoxicity for normal cells suggests that it may be efficacious for either the in vivo or in vitro killing of multiple myeloma cells.

From ImmunoGen, Inc, Cambridge, MA; and the Division of Hematologic Malignancies, Dana-Farber Cancer Institute, Boston, MA.

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Address reprint requests to Victor Goldmacher, ImmunoGen, Inc, 149 Sidney St, Cambridge, MA 02139.

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MATERIALS AND METHODS

Antibody and immunocomplex preparation. The anti-CD38 MoAb HB7 (IgG1) was developed by Dr Max D. Cooper (University of Alabama at Birmingham). The antibody was purified from ascites fluid as described previously.46 Blocked ricin was prepared and conjugated to the HB7 antibody using methods described elsewhere.46,47 Analysis by sodium-dodecyl sulfate polyacrylamide gel electrophoresis showed that the conjugate consists predominantly of a single species of apparent Mr, of 222,500 corresponding to an immunocomplex containing one molecule of blocked ricin (apparent Mr, 62,500) linked to one molecule of antibody (apparent Mr, 160,000), and that the conjugate is free of both unconjugated antibody and blocked ricin (limit of detection, <5%). The ricin A chain from the immunotoxin and from native ricin were equally potent in their ability to inhibit protein synthesis in a cell-free translation system from rabbit reticulocytes.46 The preparation and the cytokytic properties of anti-CD19-blocked ricin, anti-CD56-blocked ricin and anti-CD6-blocked ricin have been described previously.46,47 The MoAb BC-4E85 (Kabi Pharmacia, Helsingborg, Sweden) was conjugated to blocked ricin in a similar manner.

Cells. The following human cell lines were used: HS-Sultan (plasmacytoma, American Type Culture Collection [ATCC], CRL 1484); Namalwa (Burkitt's lymphoma, ATCC, CRL 1452); RPMI 8226 (myeloma, ATCC, CCL 155); U266B1 (myeloma, ATCC, TIB 196); Raji (Burkitt's lymphoma, ATCC, CCL 86); Jurkat (acute T-cell leukemia, ATCC, TIB 152); Daudi (Burkitt's lymphoma, ATCC, CCL 213); SUDHL-1 and SUDHL-10 (diffuse histiocytic lymphomas, a generous gift of Dr Alan L. Epstein [University of Southern California, Los Angeles, CA]); U-937 (histiocytic lymphoma, ATCC, CRL 1593); Molt-4 (acute lymphoblastic leukemia, ATCC, CRL 1582); HL-60 (promyelocytic leukemia, ATCC, CCL 240); ALL-3 (pre-B-acute lymphoblastic leukemia48); SW2 (small cell lung carcinoma49), and SCaBER (human bladder squamous carcinoma, ATCC, HTB 3). Cell lines were grown in RPMI-1640 medium supplemented with 10% heat-treated fetal bovine serum (FBS) or 10% iron-fortified bovine calf serum and 2 mmol/L L-glutamine, and maintained in exponential growth cultures. Peripheral blood (PB) lymphocytes were isolated by a standard Ficoll-Hypaque technique from blood freshly drawn from healthy donors. Bone marrow (BM) specimens were obtained from healthy volunteers and from multiple myeloma patients in accordance with protocols approved by the Human Subject Protection Committee of the Dana-Farber Cancer Institute. BM mononuclear cells (BMMCcs) were isolated using Ficoll-Paque (Pharmacia LKB Biotechnology, Piscataway, NJ) density gradient centrifugation technique.

Cytotoxicity assays. Cytotoxicity testing on cell lines were performed by incubating test samples with cells at 37°C for 24 hours. The cells were then washed and placed into fresh medium for measuring surviving fractions of cells directly by either the growth back extrapolation assay,50 or a clonogenic assay51 that has been modified as follows. HS-Sultan myeloma cells (4 × 10⁶ cells per mL, with or without 3.6 × 10⁴ γ-irradiated (2,500 cGy) normal BMMCs per mL) were exposed to HB7-blocked ricin for 24 hours, washed and fresh medium, and then plated in 96-well plates in RPMI-1640 medium supplemented with 5% heat-inactivated FBS.

Cytotoxicity testing on PB T cells was done by incubating test samples with resting T cells at 37°C for 24 hours. The cells were then washed and placed into fresh medium containing phytohemagglutinin, and the surviving fractions of cells were measured in a clonogenic assay using γ-irradiated autologous PB lymphocytes as feeder cells.

Inhibition of protein synthesis by BMMCs from multiple myeloma patients and healthy volunteers and by HS-Sultan and RPMI 8226 cell lines was detected by [3H]-leucine incorporation. In these experiments, cells were cultured in 96-well plates (5 × 10⁵ cells per well) in 0.1 mL of leucine-free RPMI-1640 medium supplemented with 2 mmol/L L-glutamine and 10% FCS that contained various concentrations of HB7-blocked ricin (except for the control cultures). Following 40- to 42-hour exposure to HB7-blocked ricin, the cultures were pulsed with [3H]-leucine (2 μCi per well) for 6 to 8 hours. The cells were then washed in phosphate-buffered saline (PBS) and incubated overnight with 10% aqueous solution of trichloroacetic acid at 4°C. The samples were centrifuged in an Eppendorf microcentrifuge (Brinkman Instruments, Inc, Westbury, NY) at 16,000g, the sediments were solubilized in 1 mol/L NaOH and neutralized with HCl, and the radioactivity was measured using a Betaplate liquid scintillation counter (Wallac, Gaithersburg, MD). Data were calculated as average cpm per minute (cpm) from triplicate wells, and dose-response curves were generated based on expressing cpm from immunotoxin-treated cells as a fraction of cpm from control cells.

Antibody-induced proliferation of PB T lymphocytes. E-rosette normal T cells were isolated from PB lymphocytes of healthy donors by standard techniques and incubated (10⁵ per well) in the presence of γ-irradiated autologous E-rosette feeder cells (5 × 10⁵ per well) at 37°C in a humidified atmosphere containing 5% CO2, for 3 days either in growth medium that contained the indicated antibodies or in the presence of immobilized anti-CD3 or HB7 antibody. A mitogenic combination of anti-CD2 antibodies, anti-T11, and anti-T11, was used as a positive control for proliferation induced by soluble antibody, whereas an anti-CD3 antibody, OKT3, was used as a positive control for proliferation by immobilized antibody. HB7 and OKT3 antibodies were immobilized by treating plastic plates with 0.12 mol/L antibody solution in PBS for 2 hours at 37°C and then rinsing the plates with medium. The rate of DNA synthesis was measured by incorporation of [3H]-thymidine using a procedure described elsewhere. Briefly, cultures were pulsed with [3H]-thymidine on day 3 (soluble antibodies) or day 4 (immobilized antibodies) and incorporated radioactivity measured 18 hours later. Results are expressed as the mean cpm ± SEM of triplicate cultures.

Binding analysis. Binding of HB7 antibody or HB7-blocked ricin conjugate to cells at 0°C was measured by two methods. (1) [125I]-labeled antibody was used in a direct binding assay. This binding assay was used to determine the average number of CD38 antigen molecules per cell. Radiodination of HB7 was done using the Iodo-gen technique.52 Cells were exposed to HB7 and OKT3 antibodies immobilized by treating plastic plates with 0.12 mol/L antibody solution at defined concentrations for 30 minutes at 4°C in minimum essential medium (MEM) for suspension cultures supplemented with 2.5% pooled human serum of AB type (AB buffer) and transferred to Eppendorf centrifuge tubes containing 100 μL of a silicon-oil/paraffin-oil mixture with a density of 1.009 g/mL. The cells and bound antibody were separated from unbound antibody by centrifugation; the tubes were then frozen and cut between the oil-media interface and the cell pellet. Cell-associated antibody was quantified by counting the portion of the tube containing the cell pellet in a gamma counter. Control samples contained an excess (1 μmol/L) amount of nonlabeled antibody in addition to the radiolabeled antibody: the amount of cell-associated radioactivity in these samples was negligible. (2) Binding studies by indirect immunofluorescence were done for determining values for apparent Kd (equilibrium dissociation constant). Cells (2 × 10⁵) in 100 μL of AB buffer were first incubated with nonlabeled antibody or conjugate for 30 minutes at 0°C (on ice). The cells were then washed with AB buffer and incubated for 30 minutes at 0°C with fluorescein-labeled goat-antimurine IgG (Sigma Chemical Co, St Louis, MO) at 1:50 dilution in AB buffer. The cells were washed again with AB buffer, fixed with 1% formaldehyde in 10 mmol/L potassium phosphate buffer, pH 7.2, containing NaCl (145 mmol/L) and analyzed on a flow cytometer (FACScan, Becton Dickinson, Mountain View, CA).
Table 1. Expression of CD38 and CD19 on the Surface of Various Cell Lines as Detected by Indirect Immunofluorescence Flow Cytometric Analysis With HB7 and Anti-B4 Antibody

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Origin</th>
<th>% CD38+</th>
<th>% CD19+</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS Sultan</td>
<td>Plasmacytoma/IgG multiple myeloma</td>
<td>97</td>
<td>86</td>
</tr>
<tr>
<td>RPMI 8226</td>
<td>λ light chain secreting myeloma/multiple myeloma</td>
<td>93</td>
<td>3</td>
</tr>
<tr>
<td>Namalwa</td>
<td>Burkitt’s lymphoma</td>
<td>98</td>
<td>93</td>
</tr>
<tr>
<td>ALL3</td>
<td>pre-B–acute lymphoblastic leukemia</td>
<td>94</td>
<td>89</td>
</tr>
<tr>
<td>Raji</td>
<td>Burkitt’s lymphoma</td>
<td>98</td>
<td>89</td>
</tr>
<tr>
<td>Daudi</td>
<td>Burkitt’s lymphoma</td>
<td>99</td>
<td>91</td>
</tr>
<tr>
<td>SU-DHL-1</td>
<td>Diffuse histiocytic lymphoma</td>
<td>96</td>
<td>82</td>
</tr>
<tr>
<td>SU-DHL-10</td>
<td>Diffuse histiocytic lymphoma</td>
<td>91</td>
<td>91</td>
</tr>
<tr>
<td>U266B1</td>
<td>IgE secreting myeloma/IgE myeloma</td>
<td>21</td>
<td>1</td>
</tr>
<tr>
<td>Molt-4</td>
<td>Acute lymphoblastic leukemia</td>
<td>97</td>
<td>1</td>
</tr>
<tr>
<td>Jurkat</td>
<td>Acute T-cell leukemia</td>
<td>71</td>
<td>1</td>
</tr>
<tr>
<td>HL-60</td>
<td>Promyelocytic leukemia</td>
<td>92</td>
<td>1</td>
</tr>
<tr>
<td>U-937</td>
<td>Histiocytic lymphoma</td>
<td>97</td>
<td>5</td>
</tr>
<tr>
<td>SW2</td>
<td>Small cell lung carcinoma</td>
<td>2</td>
<td>8</td>
</tr>
</tbody>
</table>

controls lacked either fluorescein-labeled antimurine IgG or the primary antibody or both. For the analysis of expression of CD38 and CD19 antigen on various cell lines, cells were incubated with a saturating concentration (0.1 μmol/L) of HB7 and anti-B4 antibody, respectively.

Treatment of normal BMMCs with HB7-blocked ricin and hematopoietic colony assays. Suspensions of BMMC (10⁵ cells in 2 mL) were treated with various concentrations of HB7-blocked ricin (10⁻⁴ to 10⁻¹⁰ mol/L) in RPM1-1640 medium containing 10% FCS for 24 hours at 37°C in a humidified atmosphere containing 5% CO₂. After toxin treatment, BMMC were washed three times with Hank’s balanced salt solution, resuspended in 1% methylcellulose (Terry Fox Laboratory, Vancouver, Canada) in Iscove’s modified Dulbecco’s medium containing FCS (25%), L-glutamine (2 mmol/L), penicillin (50 U/mL), streptomycin (50 μg/mL), granulocyte-macrophage colony-stimulating factor (GM-CSF; 400 ng/mL) (Schering-Plough Research Institute, Kenilworth, NJ), IL-3 (300 ng/mL) (Genetics Institute, Cambridge, MA), and Epogen (160 U/mL) (Amgen, Thousand Oaks, CA). Cells were plated at 3.7 × 10⁴ per well of a 24-well plate in quadruplicate. After 14 days of culture, granulocyte-macrophage colony-forming units (CFU-GM), burst-forming units–erythroid (BFU-E) and colony-forming units–granulocyte/erythroid/multilineage/macrophage (CFU-GEMM) were enumerated.

RESULTS

Binding of anti-CD38 antibody HB7 and its blocked ricin conjugate to cells. To examine the expression of CD38 by various cell lines, the binding of a saturating concentration of HB7 antibody (0.1 μmol/L) to these cells was measured by indirect immunofluorescence (Table 1). Most tested lines of B, T, and myeloid/monocytic origin had a high (≥70%) and uniform expression of CD38, with one exception; myeloma cell line U266B1 expressed the antigen only sparingly (21% positive cells). In addition, the expression of the B-cell lineage-specific antigen CD19 (antibody anti-B4) was examined (Table 1) to allow subsequent comparative analysis of HB7-blocked ricin and a highly potent anti-B4–blocked ricin. As expected, the expression of CD19 was confined to cell lines of B-cell origin. Interestingly, CD38 was expressed more uniformly than CD19 on all tested cell lines of B-cell origin.

The binding of HB7 antibody to the surface of the myeloma lines HS-Sultan, RPM1 8226, and U266B1, as well as the Burkitt’s lymphoma line Namalwa, was studied further. The average numbers of CD38 antigens per cell were determined with a saturating concentration (40 nmol/L) of [125I]-labeled HB7 antibody and were found to be 2.7 × 10⁴, 1.4 × 10⁵, 1.0 × 10⁶, and below the detection limit (10³) for the cell lines RPM1 8226, HS-Sultan, Namalwa, and U266B1, respectively. As expected, HB7 antibody showed equal avidity towards the CD38 antigen on all cell lines with an apparent Kₐ of 1.5 to 3 nmol/L (Fig 1). The avidity of HB7-blocked ricin conjugate towards CD38-expressing cells was found to be about threefold less than the avidity of the nonconjugated antibody (Fig 2).

Cytotoxicity of HB7-blocked ricin for CD38-expressing cultured cell lines. The cytotoxicity of HB7-blocked ricin on CD38-expressing Namalwa cells is shown in Table 2 and Fig 3A. For comparison, the cytotoxic effects of ricin, N901-blocked ricin (N901 is an anti-CD56 antibody; CD56 is not expressed on Namalwa cells), and of HB7-blocked ricin in the presence of an excess of HB7 antibody are also shown. Namalwa cells are very sensitive to the cytotoxic effect of HB7-blocked ricin with an IC₅₀ value of 7 × 10⁻¹² mol/L, which is almost as sensitive as to native ricin (IC₅₀ of 4.5 × 10⁻¹² mol/L). A nonbinding immunotoxin, N901-blocked
The affinity of HB7 antibody and HB7-blocked ricin to HS-Sultan cells. Cells (2 × 10⁶ per sample) were incubated with various concentrations of HB7 or HB7-blocked ricin for 30 minutes at 4°C, then with FITC-labeled goat-antimouse IgG (30 minutes at 4°C) with a wash between the incubations, and then fixed with 1% formaldehyde in PBS. Relative mean fluorescence of cells was then determined on a flow cytometer as a measure of bound antibody. Symbols represent the binding data for HB7 (●) and HB7-blocked ricin (○).

Table 2. Cytotoxicity of Ricin, HB7-Blocked Ricin, and N901-Blocked Ricin for Cell Lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>IC₅₀, mol/L After 24-h Exposure</th>
<th>Maximal Cell Kill by HB7-Blocked Ricin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ricin</td>
<td>HB7-Blocked Ricin</td>
</tr>
<tr>
<td>HS-Sultan</td>
<td>3.0 × 10⁻¹⁰</td>
<td>6.0 × 10⁻¹¹</td>
</tr>
<tr>
<td>Namalwa</td>
<td>4.5 × 10⁻¹²</td>
<td>7.0 × 10⁻¹²</td>
</tr>
<tr>
<td>RPMI 8226</td>
<td>3.0 × 10⁻¹²</td>
<td>7.0 × 10⁻¹²</td>
</tr>
<tr>
<td>U266B1</td>
<td>2.0 × 10⁻¹⁷</td>
<td>&gt;2.0 × 10⁻⁸</td>
</tr>
</tbody>
</table>

Abbreviations: SF, surviving fraction of cells; ND, not done.
* Cells were exposed to 8 × 10⁻¹⁰ mol/L HB7-blocked ricin.
† Cells were exposed to 1.5 × 10⁻¹⁰ mol/L HB7-blocked ricin. Cells were incubated with a toxin for 24 hours at 37°C in a humidified atmosphere containing 5% CO₂. Cells were then washed, placed in fresh medium, and the surviving fractions of cells were determined as outlined in Materials and Methods. The survival of U266B1 cells exposed to 0.25 nmol/L, 0.5 nmol/L, 1 nmol/L, or 2 nmol/L of HB7-blocked ricin under these conditions was 100%. IC₅₀ values were interpolated from the cytotoxicity curves as an index of the ability of the toxin to kill cells. IC₅₀ is the concentration of a toxin leaving a surviving fraction of 0.37. In a one-hit one kill model, a surviving fraction of 0.37 (1/e, where e is the natural logarithm base) is left when the number of lethal hits received by a population of cells is equal to the number of cells in that population.

The effect of CD38 antigen density on the cytotoxicity of HB7-blocked ricin was analyzed using the highly CD38⁺ multiple myeloma cell line RPMI 8226, and a myeloma cell line U266B1 that only weakly expresses CD38. The data in Table 2 show that RPMI 8226 cells are, as expected, sensitive to HB7-blocked ricin (with an IC₅₀ value of 7 × 10⁻¹⁰ mol/L), and that U266B1 cells could not be affected with concentrations of HB7-blocked ricin of up to 2 × 10⁻⁹ mol/L (the highest concentration tested). Although the cell surface phenotype of the clonogenic cell in myeloma is unknown, most malignant plasma cells strongly express CD38 in a pattern similar to that observed on RPMI 8226 cells. Malignant plasma cell depletion by HB7-blocked ricin in BM samples. Having shown that HB7-blocked ricin is highly potent and selective in killing CD38-expressing cell lines, we next determined whether this immunotoxin would be effective in killing residual malignant cells in a sample of BM. An admixture of 90% freshly isolated BM mononuclear cells and 10% HS-Sultan cells was treated with HB7-blocked ricin, and the surviving myeloma cells were detected in a clonogenic assay. As a control, HS-Sultan cells alone were treated with the immunotoxin under similar conditions (Fig 4). At the concentration of 0.3 nmol/L immunotoxin, more than two logs of these cells were depleted from the BM sample. Importantly, the degree of malignant plasma cell kill was similar in the presence and in the absence of the BM cells.

Effect of HB7-blocked ricin on normal hematopoietic stem cells. The toxicity of HB7-blocked ricin for normal hematopoietic BM clonogenic cells was evaluated to determine the safety of this immunotoxin for the ex vivo purging of tumor cells from BM. BMMCs from normal individuals were either incubated with medium or with medium containing HB7-blocked ricin at various concentrations for 24 hours, and the number of CFU-GM, BFU-E, and CFU-GEMM were then determined (Table 3). HB7-blocked ricin was only moderately toxic for these progenitors with less than 1 log depletion at concentrations (10⁻⁵ to 10⁻¹¹ mol/L) that deplete 5 to 6 logs of antigen-bearing tumor cells. This toxicity to hematopoietic stem cells is similar to that of another blocked ricin conjugate with an antibody (anti-CD33) that is not
targeted against antigens expressed by CFU-GM, BFU-E, and CFU-GEMM cells.\textsuperscript{56,57}

\textit{Inability of HB7 antibody to induce activation of normal human PB lymphocytes.} Normal human resting peripheral T cells do not express appreciable amounts of CD38 antigen on their surface.\textsuperscript{43} However, it has been reported that A10, an anti-CD38 antibody, activates human T cells.\textsuperscript{58} Such an activity would severely limit the clinical utility of HB7 antibody. Therefore, we examined whether HB7 antibody could induce activation of normal human peripheral T cells (Table 4). Resting normal PB lymphocytes were cultured with HB7 antibody under various conditions that had been previously reported as sufficient for activating T cells with A10 or other antibodies capable of activating T cells. Cells were cultured either in growth medium that contained HB7 antibody, or in plastic plates that had HB7 immobilized to their surfaces. As a positive control, cells were incubated with either a mitogenic combination of anti-CD2 antibodies, anti-T1\textsubscript{12} and anti-T1\textsubscript{13},\textsuperscript{49} or in plates that had an anti-CD3 antibody immobilized to their surfaces. As a negative control, cells were cultured in the absence of antibodies. The tritiated thymidine incorporation by the cells was then measured as an index of their activation. These experiments showed (Table 4) that, unlike the A10 antibody, HB7 does not induce activation of PB lymphocytes. However, in a manner similar to that reported for the A10 antibody,\textsuperscript{58} HB7 antibody enhances the mitogenic potential of anti-T1\textsubscript{13} antibody.

\textit{Cytotoxicity of HB7-blocked ricin for nonactivated human peripheral T cells.} We also tested whether HB7-blocked ricin was toxic for nonactivated normal human T cells (Table 5). HB7-blocked ricin was not toxic for the cells at mol/L, the highest concentration tested, consistent with the fact that nonactivated peripheral T cells do not express CD38 antigen.\textsuperscript{44} As a positive control, nonactivated T cells were exposed to anti-CF6-blocked ricin. Human T cells express this antigen on their surface and as expected,\textsuperscript{49} anti-CF6-blocked ricin was toxic for the cells.

\textit{Effect of HB7-blocked ricin on multiple myeloma cells isolated from patients.} In our attempt to test the efficacy of HB7-blocked ricin, we faced two problems. First, multiple myeloma clonogenic cells might differ from the main population of patients' multiple myeloma cells in their CD38

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{Concentration of HB7-Br (mol/L)} & \textbf{BFU-E} & \textbf{CFU-GEMM} & \textbf{CFU-GM} \\
\hline
Control & 42 ± 7 & 3.0 ± 0.3 & 52 ± 6 \\
1 × 10\textsuperscript{-11} & 48 ± 17 & 3.6 ± 1.8 & 55 ± 26 \\
5 × 10\textsuperscript{-11} & 42 ± 22 & 2.0 ± 1.0 & 37 ± 15 \\
1 × 10\textsuperscript{-10} & 20 ± 10 & 2.6 ± 1.4 & 24 ± 7 \\
5 × 10\textsuperscript{-10} & 10 ± 8 & 1.0 ± 0.6 & 9 ± 4 \\
1 × 10\textsuperscript{-9} & 3.6 ± 0.4 & 0.6 ± 0.6 & 3 ± 2 \\
5 × 10\textsuperscript{-9} & 0.5 ± 0.4 & 0 & 0 \\
1 × 10\textsuperscript{-8} & 0 & 0 & 0 \\
\hline
\end{tabular}
\caption{Cytotoxicity of HB7-Blocked Ricin for Hematopoietic Progenitors}
\end{table}

Cells were incubated with HB7-blocked ricin at the concentrations indicated for 24 hours at 37°C in a humidified atmosphere containing 5% CO\textsubscript{2}. Cells were then washed, placed in fresh medium, and the colony-forming units were enumerated as outlined in Materials and Methods. The data were obtained in three independent experiments and are presented as mean ± SEM.
expression and, therefore, in their sensitivity towards the cytotoxic effect of HB7-blocked ricin. In addition, it is difficult to reproducibly maintain multiple myeloma cells in culture for more than 2 days. Therefore, we assayed for inhibition of protein synthesis after a short-term exposure (2 days) of freshly isolated patients’ cells to immunotoxin as an index of the cytotoxic potency of HB7-blocked ricin on these cells. Multiple myeloma cells from five patients have been tested (range 20% to 70% myeloma cells) and compared with samples of normal BM from two healthy volunteers (Fig 5). CD38-expressing cell lines HS-Sultan and RPMI 8226 were used as positive controls in these experiments. Protein synthesis in cells from four patients was inhibited at low doses (between 10 and 50 pmol/L) of HB7-blocked ricin (panel A), indicating that these cells were 100- to 500-fold more sensitive than the two samples of normal BM (panel C). Cells from one patient (panel B) were more resistant than those from the other four patients towards the effects of the immunotoxin, but still appeared to be more sensitive than the normal BM. The reason for this resistance is unclear. As expected (Table 2), HS-Sultan and RPMI 8226 cells were sensitive toward HB7-blocked ricin in this test (panel D).

To determine whether the inhibition of protein synthesis induced in cells by HB7-blocked ricin was mediated via CD38 interaction, cells were exposed to a conjugate of blocked ricin with an anti-bladder carcinoma murine MoAb BC-4E8 that does not bind to cells of hematopoietic lineages. As expected, plasma cells of the myeloma patients and the myeloma cell lines were much less sensitive to this immunotoxin (open symbols; Fig 3, A, B, and D) compared with HB7-blocked ricin (corresponding closed symbols). Control experiments confirmed that BC-4E8–blocked ricin was highly potent in killing the antigen-expressing SCaBER cell line (N. Kedersha, unpublished results, March 1991).

**DISCUSSION**

We describe an immunotoxin, HB7-blocked ricin, that is selectively highly cytotoxic towards cells expressing the CD38 antigen. At concentrations that show kill of several logs of CD38+ tumor (myeloma) cells, little toxicity to hematopoietic progenitors was observed. The highly potent killing of antigen-bearing tumor cells coupled with the lack of significant effects on PB mononuclear or hematopoietic progenitor cells suggests that HB7-blocked ricin may be useful for the therapy of antigen-bearing malignancies, such as multiple myeloma.

We chose the CD38 antigen as a target because it is universally and strongly expressed on all cells among all myeloma clones obtained from patients. Most antigens other than CD38 are restricted in their expression on myeloma cells. Because it remains unclear which cells within the myeloma clones are capable of self-renewal, we chose to target the entire malignant cell population. When tested on Nalma cells, HB7-blocked ricin was comparable in potency with an anti-CD19–directed immunotoxin, anti-B4–blocked ricin, both in (1) the IC50 values (7 × 10^{-12} vs 1 × 10^{-11} pmol/L), which reflect the capacity of the immunotoxins to kill the majority of cells in the cell population; and (2) log kill (≥5 logs for both immunotoxins), which is a measure of the capacity of the immunotoxins to kill rare resistant variant cells. In our studies, the binding pattern of HB7 antibody to cell lines of B, T and myeloid/monocytic origin was similar to that described for other anti-CD38 antibodies. In particular, it was strongly expressed on myeloma, B- and T-cell acute lymphoblastic leukemias, as well as on Burkitt’s and

### Table 4. Ability of Antibodies to Activate Resting Human PB Lymphocytes

<table>
<thead>
<tr>
<th>Antibody Added to PB Lymphocytes</th>
<th>[H]dTh Incorporation, cpm × 10^3 Mean ± SEM (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (medium)</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>HB7 (60 nmol/L) in medium</td>
<td>0.35 ± 0.04</td>
</tr>
<tr>
<td>HB7 (0.3 μmol/L) in medium</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>anti-T11 (0.12 μmol/L)</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>anti-T11 (0.12 μmol/L)</td>
<td>0.50 ± 0.04</td>
</tr>
<tr>
<td>anti-T11 (0.12 μmol/L) + anti-T11 (0.12 μmol/L) in medium</td>
<td>310 ± 30</td>
</tr>
<tr>
<td>HB7 (60 nmol/L) + anti-T11 (0.12 μmol/L)</td>
<td>0.53 ± 0.09</td>
</tr>
<tr>
<td>HB7 (60 nmol/L) + anti-T11 (0.12 μmol/L) in medium</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>HB7 (0.3 μmol/L) + anti-T11 (0.12 μmol/L)</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>HB7 (0.3 μmol/L) + anti-T11 (0.12 μmol/L) in medium</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td>HB7-immobilized</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>anti-CD3 adhered</td>
<td>88 ± 4</td>
</tr>
</tbody>
</table>

*E*-rosette* normal T cells were cultured (10^6 per well) in the presence of irradiated autologous *E*-rosette* feeder cells (5 × 10^6 per well) at 37°C in a humidified atmosphere containing 5% CO2 for 3 days either in growth medium that contained the indicated antibodies or in the presence of immobilized anti-CD3 or HB7. A mitogenic combination of anti-CD2 antibodies anti-T11 and anti-T11 was used as a positive control for proliferation induced by soluble antibody, whereas anti-CD3 (OKT3) was used as a positive control for proliferation by immobilized antibody. Cultures were pulsed with [3H]-thymidine on day 3 (soluble antibodies) or day 4 (immobilized antibodies) and incorporated radioactivity measured 18 hours later. Results are expressed as the mean cpm ± SE of triplicate cultures.

### Table 5. Cytotoxicity of HB7-Blocked Ricin for Normal Resting Human Peripheral T Cells

<table>
<thead>
<tr>
<th>Agent</th>
<th>Plating Efficiency*</th>
<th>Surviving Fraction of Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium only</td>
<td>0.29</td>
<td>1.00</td>
</tr>
<tr>
<td>Anti-CD6–blocked ricin (1 nmol/L), a positive control</td>
<td>0.028</td>
<td>0.094</td>
</tr>
<tr>
<td>HB7-blocked ricin (0.1 nmol/L)</td>
<td>0.37</td>
<td>1.26</td>
</tr>
<tr>
<td>HB7-blocked ricin (1 nmol/L)</td>
<td>0.26</td>
<td>0.89</td>
</tr>
<tr>
<td>HB7-blocked ricin (1 nmol/L) + HB7 (100 nmol/L)</td>
<td>0.39</td>
<td>1.33</td>
</tr>
</tbody>
</table>

Freshly isolated normal resting PB lymphocytes were incubated in growth medium with immunotoxin at 37°C for 24 hours, and then washed.

* Plating efficiency is a fraction of plated cells that form colonies. Plating efficiencies and the surviving fractions of cells were established in a clonogenic assay as described in Materials and Methods.
Inhibition of protein synthesis in patients’ multiple myeloma cells and normal BM cells. BMMCs from multiple myeloma patients and healthy volunteers were used in these experiments. Inhibition of protein synthesis was detected by \(^{3}H\)-leucine incorporation during an 8-hour pulse after 40-hour exposure of cells to HB7-blocked ricin (filled symbols) or a nonbinding immunotoxin, BC-4E8-blocked ricin (open symbols). The results for patients’ multiple myeloma cells are shown in A and B, for normal BM cells in C, and for multiple myeloma-derived cell lines HS-Sultan (\(\bullet\)) and RPMI 8226 (\(\square\)) in D. Symbols in A represent patient 1 (triangles), 3 (squares), 4 (circles), and 5 (diamonds), respectively. B represents patient 2. BM from patients 1, 2, 3, 4, and 5 contained 70%, 40%, 50%, 50%, and 20% myeloma cells, respectively. Samples 1 and 2 had been previously frozen and were thawed immediately before the experiment.

Some non-Hodgkins lymphomas. Because this antigen is found on malignant cells in virtually all patients suffering from multiple myeloma, such an immunotoxin may be an efficacious agent for treatment of this disease. Nonetheless, because the phenotype of the clonogenic cell in myeloma is undefined and theoretically this cell could lack surface CD38 expression, the therapeutic value of HB7-blocked ricin remains to be defined in clinical studies.

Normal resting peripheral T cells do not express appreciable amounts of CD38 antigen, suggesting that HB7-blocked ricin should not cause any adverse effects on these cells. Indeed, HB7-blocked ricin does not kill resting T cells, which lack CD38 expression. The A10 MoAb, also directed at the CD38 antigen, can augment proliferation of normal human T cells and lines expressing CD38 independently of accessory cells or IL-2. Therefore, we similarly examined HB7 MoAb and our studies showed that HB7 antibody does not induce T-cell activation; our previous studies have shown that HB7 does not cause proliferation of myeloma cell lines.\(^{17}\) However, HB7 antibody has a weak comitogenic activity toward T cells when applied together with anti-T11.\(^{17}\) The mechanism of this phenomenon and its in vivo significance is not clear.

HB7-blocked ricin showed limited toxicity for normal hematopoietic progenitors of the BM. Experiments with admixtures of a tumor cell line with normal BMMCs showed more than 2 logs of tumor cell kill under conditions that were not very toxic to the marrow. This toxicity appears to be nonspecific because it is in the same range as the one observed for anti-My9—blocked ricin (anti-My9 is an anti-CD33 antibody),\(^{6,5}\) an immunotoxin that binds to an antigen not expressed on BM clonogenic cells. This would confirm previous reports that hematopoietic stem cells do not express CD38 antigen. Indeed, hematopoietic colonies from CD34^+CD38^- progenitor cells have been generated that are consistent with the view that depletion of CD38^+ cells may not deplete nonlineage committed CD34^+CD38^- progenitor cells.\(^{6,6}\) Thus, HB7-blocked ricin appears to be safe as a BM purging agent.

In addition to multiple myeloma, anti-CD38-directed blocked-ricin conjugates may also have potential for a number of other therapeutic applications that involve eradication of CD38^+ cells such as lymphomas and leukemias, as well as in vivo (systemic) and ex vivo suppression of autoimmune disease by killing activated T cells.

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The authors thank Dr Max D. Cooper, University of Alabama at Birmingham, for the generous gift of the HB7 antibody; our colleagues at ImmunoGen, Richard Cornell and Carlos A. Acevedo, for the preparation of HB7-blocked—ricin conjugate; Margaret Johnson and Tammy Davis for expert technical assistance; and Dr Walter A. Blüttler (ImmunoGen, Inc) for critical reading of the manuscript.

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Anti-CD38-blocked ricin: an immunotoxin for the treatment of multiple myeloma [see comments]

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