Use of an Antibody–Ricin A-Chain Conjugate to Delete Neoplastic B Cells From Human Bone Marrow

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Affinity-purified rabbit antibody to human lambda and kappa chains (RaHXx) was conjugated to the A-chain of the plant toxin, ricin. The resulting immunotoxin (RaHXx-A) killed cells from the tumor cell line Daudi, which bears surface immunoglobulin, but was nontoxic to the CFU-E, BFU-E, and CFU-GM of human bone marrow. RaHXx-A eliminated 99% of clonogenic Daudi cells that had been mixed with marrow cells in vitro, without demonstrable toxicity to hematopoietic cells. Thus, in vitro treatment of marrow with RaHXx-A may increase the incidence of cure following autologous bone marrow transplantation for the treatment of human B-cell malignancies.

Antibodies conjugated to intact ricin or its A-chain are specifically toxic under appropriate in vitro conditions to cells bearing relevant target antigens. Thus, antibody–toxin conjugates (immunotoxins) can kill virtually all T or B cells present in in vitro suspensions of rodent bone marrow without compromising the marrow’s capacity to reconstitute lethally irradiated recipients.1–3 Similar conjugates with specificity for human cells have been reported,4–7 but their effects on normal human bone marrow have not been described. In this article, we describe an antibody–ricin A-chain conjugate that kills the human lymphoblastoid B-cell line, Daudi, but is harmless to colony-forming cells in normal human marrow. This and similar reagents may be useful for eliminating neoplastic cells from bone marrow in conjunction with autologous bone marrow transplantation for the treatment of human malignancies.

MATERIALS AND METHODS

Antibodies

Human IgG was prepared from the serum of normal human donors. Its purity was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Rabbis were immunized with 50 μg of the purified IgG in complete Freund’s adjuvant and were boosted 1 mo later. Pooled sera were affinity-purified on Sepharose-IgG using 1 vol of packed Sepharose/10 ml of antiserum. After the gel matrix was washed, the antibody was eluted in 3.5M MgCl₂. The eluate was dialyzed against water and then phosphate-buffered saline (PBS) and concentrated to 1 mg/ml by vacuum dialysis. As assessed by radioimmunoassay (RIA), the affinity-purified antibody reacted with human γ, ε, and λ chains. The affinity-purified rabbit antiovalbumin (RaOVA) and goat anti-rabbit Ig (GAR Ig) were prepared as described previously.

Ricin A-Chain

Ricin A-chain was purchased from XOMA Corporation (San Francisco, CA). Ricin A-chain was prepared by reduction of whole ricin and purified on a series of affinity columns. The A-chain was stored in a stabilizing medium to ensure retention of function during long-term storage. Four parameters were used to monitor the purity of the ricin A-chain preparation. Isoelectric focusing revealed two isomers with a major form at pl 7.5 and a minor form at 7.2. A pl of 7.3 for ricin A-chain has been previously reported.8,9 SDS-PAGE also showed two isomers, a major form having a molecular weight of 30,000 and a minor form of 34,000 in accordance with previous reports.9,11 Isoelectric focusing and SDS-PAGE revealed no evidence of ricin B-chain, whole ricin, or ricin agglutinin. Two biologic assays were used to determine the purity and the enzymatic activity of the ricin A-chain preparation. A whole cell toxicity assay, similar to that described previously,12 revealed an approximately 5 log lower toxicity of ricin A-chain compared to whole toxin. The reticulocyte lysis assay, a cell-free protein synthesis system also described previously,13 demonstrated potent inhibition of protein synthesis. Absence of whole ricin was further demonstrated by injecting 1 mg of ricin A-chain into mice, which resulted in no observable toxicity. This compares favorably with previously reported in vivo results.14

Antibody–Ricin A-Chain Immunotoxins

RaHXx and RaOVA were conjugated to ricin A-chain as described previously.15 Briefly, the antibodies were treated with the heterobifunctional crosslinker N-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP). The PDP-derivatized antibodies were then mixed with ricin A-chain at pH 7.8, and the resulting conjugate was separated from free A-chain by gel filtration on Sephacryl 200. Finally, a carrier protein consisting of reduced and alkylated rabbit alpha globulin (PENTEX Biochemicals, Kankakee, IL) was added to each conjugate preparation to a final concentration of 1 mg/ml. Such conjugates were stable for at least 6 mo at 4°C. The antibody–A-chain conjugate preparations containing RaHXx and RaOVA will be referred to as RaHXx-A and RaOVA-A, respectively. The final concentration of antibody in each preparation was adjusted to 50 μg/ml, as determined by radioimmunoassay.

Daudi Cells

Daudi is a human lymphoblastoid B-cell line obtained from a Burkitt’s lymphoma.16 The Daudi cells were passaged in tissue culture medium consisting of RPMI 1640 (MA Bioproducts, Walkersville, MD) supplemented with 10% fetal calf serum (GIBCO, Grand Island, NY), 1-glutamine, 5 x 10⁻³ M 2-mercaptoethanol, and antibiotics as previously described.17 Indirect immunofluorescence, using RaHXx as the primary antibody and fluorescein-conjugated GAR Ig as secondary antibody, confirmed that 100% of
the Daudi cells bear surface membrane Ig, which is known to be IgM. No immunofluorescence was detected when RaOVA was used as the primary antibody in this assay.

Normal Human Bone Marrow

Volunteer donors gave informed consent according to guidelines of the Human Subjects Committee at both the University of Texas and the Hutchinson Cancer Research Center. Bone marrow was aspirated from the posterior iliac crest and the mononuclear cell fraction was isolated from Ficoll-Isopaque gradients or from centrifuged buffy coats.

Treatment of Cells With RaHx-A and RaOVA-A

Daudi cells and bone marrow cells were washed and suspended in Hanks’ balanced salt solution (HBSS) or PBS. For the mixture experiments, 3 single cell suspensions were prepared in parallel: Daudi cells alone, bone marrow cells alone, and Daudi and bone marrow cells mixed in a ratio of 1:20. The cells were treated with either HBSS or PBS alone (“no treatment”), RaHx-A, or RaOVA-A. The final concentration of each cell suspension containing buffer alone, RaHx-A, or RaOVA-A was always 10^6/ml, and the cells were incubated for 20 min at 4°C. Treatment was terminated by washing the cells twice.

Amino Acid Incorporation

The procedure for assaying [3H]-leucine incorporation by cells in tissue culture has been described. Briefly, 2 x 10^6 Daudi cells/microtiter well were treated with the appropriate dilution of RaHx-A or RaOVA-A in HBSS or PBS for 20 min at 4°C. Each cell was then washed 3 times in HBSS or PBS, suspended in leucine-free tissue culture medium, and cultured for 24 hr at 37°C. Each well was then pulsed with 1 μCi of [3H]-leucine (Amersham, Arlington Heights, IL), and the cells were harvested 6–24 hr later on a Mash apparatus. Incorporation of leucine was quantitated by scintillation counting.

Soft Agar Cloning

Daudi cell clones and bone marrow CFU-GM. In vitro growth of granulocyte-macrophage colonies (CFU-GM) from bone marrow requires the constant presence of colony-stimulating activity (CSA) in the tissue culture medium. For all experiments involving parallel cultures of Daudi and bone marrow cells in soft agar, giant cell tumor-conditioned medium (GCT) from Gibco was used as the source of CSA. In these experiments, tissue culture medium was the same as that used for passage of Daudi cells, except for the addition of 10% GCT and 0.3% Noble Agar (Difco, Detroit, MI). An aliquot of each treated cell suspension was added to the agar-medium at 42°C and poured into 60 × 15 mm dishes with 2-mm grids (Costar, Cambridge, MA) in a final volume of 3 ml. Each dish received 2.5 x 10^9 Daudi cells, 5 x 10^1 bone marrow cells, or 2.5 x 10^8 Daudi cells plus 5 x 10^7 bone marrow cells. The cells were cultured under 5% CO2 at 37°C. For all experiments involving parallel or mixed cultures of Daudi and bone marrow cells, colony counts were determined after 8 days of culture.

Colonies were visualized under an inverted phase contrast microscope. Only CFU-GM containing more than 50 cells were counted as “colonies,” and the same standard was adopted for the Daudi cells. Daudi and bone marrow colonies were distinguished in the coculture experiments on the basis of colonial morphology, as described in Results. Colony counts per dish were based on a survey of at least 100 grids per dish. Since the frequency of Daudi cell colonies in cultures of cell suspensions pretreated with RaHx-A was low, the entire dish was surveyed to determine the number of Daudi colonies in these particular cultures.

Bone marrow CFU-E, BFU-E, and CFU-GM were assayed in parallel with thymidine incorporation by Daudi cells. The effect of RaOVA-A and RaHx-A on erythroid as well as granulocyte-macrophage colonies was assayed in a separate group of experiments. In these experiments, leucine incorporation by Daudi cells grown in microtiter wells and pretreated with either medium alone or RaHx-A was assayed to verify toxicity of the antibody conjugate for the appropriate target cell.

Erythroid burst-forming units (BFU-E) and erythroid colony-forming units (CFU-E) were grown in a plasma clot culture system consisting of Alpha plus medium, 30% FCS, 10% bovine serum albumin (Fraction V, Sigma Chemical Co., St. Louis, MO), 10% beef embryo extract (GIBCO, Grand Island, NY), 10^-4 M 2-ME, 70 μg/ml CaCl2, 100 U/ml penicillin G, 100 μg/ml streptomycin, 10% citrated bovine plasma (Colorado Serum, Denver, CO), and partially purified human urinary erythropoietin (0.1 U/ml for CFU-E and 2.0 IU/ml for BFU-E) (Medium Preparation Services, Vancouver, B.C.). Conditioned medium from the Mo cell line was added as a source of burst-promoting activity. Cells were cultured at 1–2 x 10^4/ml in 150-μl aliquots in wells of flexible microtiter plates (Dynatech Laboratories, Alexandria, VA) at 37°C, 5% CO2, 100% humidity. CFU-E were harvested at day 5 and BFU-E at day 14. Clots were dried and fixed with 5% glutaraldehyde onto microscope slides and then stained to identify hemoglobin-containing cells. Clusters of four or more subcolonies containing 50 or more mature erythroblasts were counted as 1 BFU-E. CFU-E were identified as single colonies with 8–64 mature erythroblasts.

In the experiments in which CFU-GM were cultured in parallel with erythroid colonies, culture conditions for the CFU-GM were somewhat different from those previously described for the Daudi- or bone-marrow mixing experiments. Bone marrow cells in Alpha medium were added to 0.8% methylcellulose in Iscove’s medium containing 1% bovine serum albumin, 30% pooled male human serum, 100 U/ml penicillin G, and 100 μg/ml streptomycin. Conditioned medium from the Mo cell line and unconditioned human placenta-conditioned medium prepared according to the methods of Schlunk and Schleyer were added as a source of CSA. Cells were cultured at 1–2 x 10^4/ml in 35-mm Petri dishes, and CFU-GM were enumerated after 14 days in culture.

RESULTS

Effect of RaHx-A on Protein Synthesis in Daudi Cells

One hundred percent of the Daudi cells displayed surface membrane fluorescence in an indirect immunofluorescence assay using RaHx (data not shown). In contrast, no fluorescence was detected using RaOVA. The capacity of RaHx-A to inhibit protein synthesis in Daudi cells is shown in Table 1. At 3 μg/ml and 12 μg/ml of RaHx-A, [3H]-leucine incorporation was inhibited by >50% and >97%, respectively. In contrast, RaOVA-A did not inhibit protein synthesis at any concentration tested.

Effect of RaHx-A on Daudi Cell Cloning in Soft Agar

Measurement of [3H]-leucine incorporation does not permit a precise determination of the frequency at
which target cells escape killing. Therefore, we also tested the effect of RaH\(\alpha\)-A on the growth of Daudi cells in a clonal assay system. Only colonies containing at least 50 Daudi cells were enumerated in this assay. Although smaller clusters and single cells were also present after 8 days of culture, cells that had not entered into cycle by 4-5 days did not proliferate during the period of observation. Furthermore, in preliminary experiments, we had determined that the numbers of clusters in immunotoxin-treated cultures did not change between days 8 and 12.

In the experiment depicted in Table 2, the cloning frequency of untreated Daudi cells was 2%. Treatment with RaH\(\alpha\)-A at a dilution of 3 \(\mu\)g/ml reduced the number of clones by about 97%. Higher concentrations of RaH\(\alpha\)-A killed 100% of the cells. In contrast, RaOVA-A had no effect on the cloning frequency at the highest concentration tested.

Absence of Toxicity of RaH\(\alpha\)-A for Bone Marrow CFU-E, BFU-E, and CFU-GM

The above data suggest that RaH\(\alpha\)-A is specifically toxic to surface Ig-bearing cells. However, cells lacking surface Ig were not present in the treated cell suspensions. To confirm this conclusion, we devised two sets of experiments to show that RaH\(\alpha\)-A is specifically toxic to Ig-bearing cells. First, human bone marrow treated with RaH\(\alpha\)-A was assayed for CFU-E, BFU-E, and CFU-GM. In the second set of experiments, a suspension containing both bone marrow and Daudi cells was treated with RaH\(\alpha\)-A to ascertain whether the conjugate could specifically kill the appropriate target cell in a mixture of cells.

Table 3 shows the results of the first set of experiments. A concentration of RaH\(\alpha\)-A that resulted in 97% inhibition of leucine incorporation by Daudi cells had no toxic effect on CFU-E, BFU-E, or CFU-GM. RaOVA-A was similarly nontoxic.

Effect of RaH\(\alpha\)-A on a Mixture of Daudi and Bone Marrow Cells

Preliminary experiments in which Daudi and human bone marrow cells were cultured separately or together in agar had revealed that Daudi cell colonies and marrow CFU-GM could be distinguished on the basis of colonial morphology. CFU-GM in agar generally display some degree of cell dispersion. Colonies of eosinophils show considerably less dispersion but are infrequent at 8 days. Daudi cell colonies have a “beehive” appearance, as if the cells were embedded in a dense matrix (Fig. 1).

Bone marrow aspirates from three normal volunteers were used for three successive mixing experiments. Only CFU-GM were assayed from the bone marrow in these experiments. In the first experiment, immunotoxins were used at a concentration of 12 \(\mu\)g/ml. In the subsequent experiments, immunotoxins were used at a concentration of 25 \(\mu\)g/ml. Each of the mixing experiments generated two sets of data, depicted by Tables 4 and 5, respectively. Thus, each time Daudi cells and bone marrow cells were cocultured, the same cell populations were also treated and cloned separately; for clarity, these latter results are displayed separately in Table 4. Note that the number

Table 3. Soft Agar Cloning of Bone Marrow Cells Following Treatment With Immunotoxins

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CFU-E</th>
<th>BFU-E</th>
<th>CFU-GM</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>144 ± 3</td>
<td>86 ± 3</td>
<td>44 ± 3</td>
</tr>
<tr>
<td>RaOVA</td>
<td>210 ± 19</td>
<td>79 ± 3</td>
<td>57 ± 3</td>
</tr>
<tr>
<td>RaOVA-A+</td>
<td>189 ± 20</td>
<td>97 ± 3</td>
<td>51 ± 4</td>
</tr>
<tr>
<td>RaH(\alpha)-A+</td>
<td>272 ± 12</td>
<td>95 ± 7</td>
<td>43 ± 3</td>
</tr>
</tbody>
</table>

*Numbers represent growth (mean ± SEM/10\(^{6}\) marrow cells) in 6 replicate cultures of 0.6 \(\times\) 10\(^{5}\) cells for BFU-E and CFU-E or 5 replicate cultures of 10\(^{6}\) cells for CFU-GM. This is 1 of 3 representative experiments.

†12 \(\mu\)g/ml. This concentration of RaH\(\alpha\)-A reduced Daudi cell incorporation of \(^3\)H-leucine by 97%.
of Daudi cell colonies per $2.5 \times 10^4$ untreated input cells varied tremendously from experiment to experiment (range of cloning frequency 0.6%-18.2%), but triplicate cultures in any given experiment gave very consistent results. The effect of RaOVA-A on unmixed Daudi cells was variable, ranging from a 30% decrease in cloning frequency (experiment 3) to a more than twofold increase (experiment 2, Table 4). Compared to either no treatment or treatment with RaOVA-A, treatment of unmixed Daudi cells with RaHx\textsubscript{x}-A gave a >99% reduction of colonies in all three experiments (Table 4). In contrast, RaHx\textsubscript{x}-A augmented the number of granulocyte-macrophage colonies from unmixed bone marrow (Table 4). The effect of RaOVA-A on bone marrow CFU-GM was variable, but it was not toxic in any of the experiments.

Table 5 shows the results obtained by mixing Daudi cells with marrow cells from these same donors prior to treatment with antibody--A-chain conjugates. The results are entirely consistent with the data from the unmixed cultures. RaOVA-A augmented the cloning efficiency of the Daudi cells in all three mixed cultures. If this number is taken as the control value, then RaHx\textsubscript{x}-A reduced the number of Daudi cell colonies in mixed cultures from the three experiments by 97%, 99%, and 99%, respectively. Killing of Daudi cells by RaHx\textsubscript{x}-A was slightly less efficient in mixed cultures than in unmixed cultures. Since clonal elimination in the latter approached 100%, this difference cannot be explained by phenotypic variants of the Daudi cells lacking surface immunoglobulin. Both RaOVA-A and RaHx\textsubscript{x}-A increased the number of CFU-GM in mixed cultures. This increase is most likely a nonspecific effect of the antibody--A-chain preparations. Thus, RaHx\textsubscript{x}-A eliminated 99% of clonogenic Daudi cells but had no effect on CFU-GM in a cell suspension containing both.

**DISCUSSION**

The present studies extend previous findings in the rodent\textsuperscript{1-3} that the A-chain of ricin conjugated to a tumor-reactive antibody can kill neoplastic B cells in tumor-infiltrated bone marrow but is not highly toxic to hematopoietic stem cells. Thus, treatment of a mixture of Burkitt lymphoma cells (from the cell line Daudi) and normal human marrow with A-chain conjugated to rabbit anti-human Ig resulted in almost complete killing of the tumor cells, with notable lack of toxicity to erythroid and myeloid progenitor cells. The use of a clonal assay allowed a precise determination of the number of target cells deleted and demonstrated

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**Table 4. Soft Agar Cloning of Parallel Cultures of Daudi Cells and Bone Marrow Cells Following Treatment with Immunoxin**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cells</th>
<th>Treatment</th>
<th>Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Daudi</td>
<td>None</td>
<td>4,550</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RaOVA-A</td>
<td>4,970 ± 737</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RaHx\textsubscript{x}-A</td>
<td>36 ± 24</td>
</tr>
<tr>
<td></td>
<td>BM‡</td>
<td>None</td>
<td>168 ± 40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RaOVA-A</td>
<td>268 ± 63</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RaHx\textsubscript{x}-A</td>
<td>248 ± 42</td>
</tr>
<tr>
<td>2</td>
<td>Daudi</td>
<td>None</td>
<td>138</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RaOVA-A</td>
<td>330</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RaHx\textsubscript{x}-A</td>
<td>1 ± 1</td>
</tr>
<tr>
<td></td>
<td>BM‡</td>
<td>None</td>
<td>359 ± 64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RaOVA-A</td>
<td>359 ± 33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RaHx\textsubscript{x}-A</td>
<td>457 ± 21</td>
</tr>
<tr>
<td>3</td>
<td>Daudi</td>
<td>None</td>
<td>1,010 ± 277</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RaOVA-A</td>
<td>698 ± 73</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RaHx\textsubscript{x}-A</td>
<td>0 ± 0</td>
</tr>
<tr>
<td></td>
<td>BM‡</td>
<td>None</td>
<td>370 ± 83</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RaOVA-A</td>
<td>399 ± 116</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RaHx\textsubscript{x}-A</td>
<td>677 ± 53</td>
</tr>
</tbody>
</table>

*2.5 \times 10^4 Daudi cells or 5 \times 10^5 bone marrow cells were plated out in soft agar in each dish. The numbers listed represent the mean colony counts per dish obtained from each treatment group. Most of the treatment groups contained 3 dishes that were counted for colonies, and the mean colony count ± SD is given. Otherwise, the mean count from 2 dishes is given. In the first experiment, RaOVA-A and RaHx\textsubscript{x}-A were used in a concentration of 12 µg/ml. In experiments 2 and 3, the concentration of antibody--A-chain was 25 µg/ml.*

†Bone marrow assayed for CFU-GM exclusively.

‡Each experiment utilized a different bone marrow donor.
that approximately 99% of the tumor cells capable of forming colonies were killed under the conditions employed, i.e., 12–25 µg of conjugate/ml for 20 min at 4°C.

There are few published data regarding the nonspecific toxicity of antibody–ricin conjugates on normal human tissue and no report defining such toxicity by a clonal assay. Using normal human blood mononuclear cells, Volkman et al. 7 recently demonstrated that a tetanus toxoid–ricin conjugate reduced pokeweed-mitogen-induced antitoxoid production, but did not affect either anti-KLH or total immunoglobulin synthesis.

In vitro culture of bone marrow has defined certain erythroid and myeloid progenitor cells (CFU-E, BFU-E, and CFU-GM). However, there is no assay in man for the pluripotent or totipotent stem cell presumably essential for reconstitution of hematopoietic tissues following lethal irradiation. However, it is plausible to speculate that in vitro incubation with antibody–A-chain conjugates will prove harmless to these cells as well. Thus, ricin A-chain conjugated to antibody against human immunoglobulin may eradicate neoplastic B cells in marrow taken from a patient with a B-cell malignancy without compromising the marrow’s capacity to reconstitute hematopoietic tissues following lethal irradiation and/or chemotherapy.

Bone marrow has also been treated in vitro with antibody and complement (C)’ prior to autologous transplantation. For example, Ritz et al. 27 used a monoclonal antibody to the common acute lymphoblastic leukemia antigen (CALLA) and rabbit C’ to kill leukemia cells in marrow taken from patients with ALL. The patients were transplanted with autologous marrow after receiving lethal chemotherapy/radiation, and marrow engraftment occurred in all four patients. Two of the four have remained disease-free for more than a year, suggesting that the procedure eradicated malignant cells in the treated marrow.

The present studies performed with a human neoplastic cell line do not necessarily imply that anti-immunoglobulin will suffice to kill neoplastic cells in the bone marrow from a patient with a spontaneous B-cell tumor. Since cells earlier in the B-cell lineage have been implicated in the neoplastic process in the human,28 it may be essential to use immunotoxins of monoclonal antibodies against B-cell differentiation antigens, including those antigens expressed on pre-B cells. Thus, one might visualize a “cocktail” of antibodies against an array of markers on the B-cell lineage as offering the greatest likelihood of killing all neoplastic cells in the marrow.

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Use of an antibody-ricin A-chain conjugate to delete neoplastic B cells from human bone marrow

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