Anti-B4–Blocked Ricin Synergizes With Doxorubicin and Etoposide on Multidrug-Resistant and Drug-Sensitive Tumors

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Anti-B4–blocked ricin (anti-B4–bR) is an immunotoxin directed against CD19-positive cells that is currently being tested in several B-cell leukemia/lymphoma clinical trials. To explore the possibility of using anti-B4–bR in combination with chemotherapy protocols, we investigated the in vitro and in vivo cytotoxic effects of combining it with doxorubicin or etoposide using the lymphoma cell line Namalwa and a P-glycoprotein–expressing cell line, Namalwa/mdr-1, obtained by retroviral infection of Namalwa cells with the mdr-1 gene. Namalwa/mdr-1 cells were slightly more sensitive to anti-B4–bR than Namalwa cells; IC50 values were approximately 4 pmol/L and 8 pmol/L, respectively. When anti-B4–bR was combined simultaneously with doxorubicin or etoposide, additive to supra-additive killing of Namalwa and Namalwa/mdr-1 cells was observed. In xenografts of Namalwa/mdr-1 cells in severe combined immunodeficiency (SCID) mice, doxorubicin and etoposide at their maximum tolerated doses (3 mg/kg × 3 or 15 mg/kg × 3) showed no therapeutic effect. However, treatment with 5 daily bolus injections of anti-B4–bR (50 μg/kg) followed by treatment with doxorubicin or etoposide significantly increased the life span of the mice by 129% and 115%, respectively. After treatment with anti-B4–bR, the Namalwa/mdr-1 population expressed lower levels of P-glycoprotein, and this decrease may account for the synergistic action of the drug combinations. These results suggest that anti-B4–bR could be used to good effect in combination with current treatment regimens and further hint at a promising role for this immunotoxin in treatment of disease at the minimal residual disease stage, where cells may be resistant to chemotherapy. © 1995 by The American Society of Hematology.
as an activated analog of cyclophosphamide, was purchased from Nova Pharmaceutical Corporation (Baltimore, MD). Anti-B4–br (Oncolyin B) was manufactured at ImmunoGen (Cambridge, MA).

Construction of mdr-1 plasmid, transfection of DOL–mdr virus producer cells, and selection of DOL–mdr virus producer cells. The human DNA for mdr (provided by Dr P. Borst, The Netherlands Cancer Institute, Amsterdam, The Netherlands) was cloned into the BanHI-Sal I cloning site of the retrovirus expression vector DOL (within the transcriptional unit of the recombinant provirus) to make pDOL–mdr. The retrovirus expression vector pDOL–mdr was introduced into the amphotrophic retrovirus packaging cell line CRIP by transfection, and transfected cells were selected in G418 (0.8 mg/mL) in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS). Colonies of G418 transfecants were pooled, and recombinant retrovirus was harvested from cell supernatants and tested for transmission of mdr-1 by infecting NIH 3T3 cells.

Infection of Namalwa cell line with retrovirus containing mdr-1 gene. Namalwa cells (3 × 10^5) were incubated with 40 mL of RPMI 1640 medium containing 10% FBS supplemented with 75% filtered (0.2 μm filter) cell culture supernatant from CRIP-producer cell line in the presence of 100 ng/mL polybrene for 24 hours. Cells were pelleted, resuspended in fresh medium, and plated at 2 × 10^5/mL in 24-well plates. Vincristine was added to a final concentration of 50 nM/mL, 75 nM/mL, or 100 nM/mL. Medium and drug were replenished every 4 days. All cell lines were maintained in RPMI 1640 medium containing 10% FBS at 37°C and 5% CO₂ in a humid atmosphere.

Southern blot analysis. Genomic DNA was isolated from 10⁷ cells, digested to completion with Kpn I, subjected to electrophoresis in 1% agarose gels, and transferred to nylon membranes as described. There is a single Kpn I site at the 3′ end of human mdr-1 cDNA and one additional site in each of the long terminal repeats of the recombinant provirus in the DOL– expression vector. Blots were probed with 32P-labeled insert DNA from the pDOL–mdr vector prepared by oligonucleotide labeling.

Indirect immunofluorescence. Cells in RPMI 1640 medium containing 10% pooled human AB serum were incubated with murine monoclonal antibody anti-B4 (IgG1; ImmunoGen) or an antibody 4E3 (IgG2A) against a surface epitope of P-glycoprotein at a final concentration of 10⁻⁴ mol/L for 30 minutes at 20°C, then washed three times and exposed to fluorescein-labeled (Fab’2), fragments of goat Ig to mouse IgG for 30 minutes at 4°C. Cells were again washed twice, and the cell-associated fluorescence was quantified using a FACSscan flow cytometer (Becton Dickinson, San Jose, CA).

Cytotoxicity assays. Cytotoxicity of anti-B4–br and drugs was evaluated using a tritiated thymidine ([³H]TdR) incorporation assay. Cells were seeded in the presence of immunotoxin and/or drugs in 96-well, flat-bottom plates for 72 hours, followed by a pulse with [³H]TdR, 1 μCi per well, for 18 hours. Cells were harvested onto fiber glass filters using a 96-well pitate cell harvester (Tomtec, Orange, CT) and counted in a Beta Plate liquid scintillation counter (Wallac, Turku, Finland). Data were calculated as average counts per minute (cpm) from triplicate wells in triplicate plates, and dose response curves for drugs were generated based on expressing cpm from treated cells as a fraction of cpm from untreated cells. Each experiment was performed three times. Before the experiments, the two cell lines were titrated to determine optimum numbers of cells per well for maximum cpm incorporated in the control: 1.7 × 10⁵ cells per well for Namalwa and 1.2 × 10⁶ cells per well for Namalwa/mdr-1, respectively.

Data analysis. Data were analyzed using an adaptation of isobologram methodology for the case where one agent is held constant. The data for dose response curves for each drug alone were entered into the computer. For each concentration of immunotoxin, envelopes of additive effect were generated with varying doses of each drug. Envelopes of additivity are derived from mode I additivity, which accounts for potency differences between the two agents, and mode II additivity, which is derived from the linear isoeffect relationship. Combinations producing an effect within the envelope boundaries of mode I and mode II are considered to be additive; those displaced to the left are greater than additive (ie, supra-additive), whereas those displaced to the right are less than additive (ie, subadditive). In the special case where the level of one agent is held constant, an isobologram can be derived that plots the expected effects (mode I and mode II) for any level of the variable agent combined with the fixed agent. Experimentally, this approach is simple and facilitates the determination of additive and nonadditive combinations. The computer program first deduces the best-fitting dose response curves using dose (or log dose) and effect, probit percentage effect, or logit percentage effect relationships. For dose response curves, correlations of ≥0.96 have been obtained. The program then calculates an isobologram at a constant level of the selected agent.

Animal and tumor model. Female CB-17 SCID mice were obtained from Massachusetts General Hospital (Boston, MA) and were maintained in a pathogen-free environment with food and water available at will. The tumor model for Namalwa cells was previously described. Briefly, when the mice were 6 to 7 weeks old, a suspension of Namalwa or Namalwa/mdr-1 cells (4 × 10⁵ cells in 0.2 mL) was injected into a lateral tail vein of animals. This procedure resulted in 100% tumor engraftment, with untreated or phosphate-buffered saline (PBS)-treated animals dying over an approximately 5-day range and with a median survival time (MST) of about 25 days after tumor inoculation.

Treatment techniques, endpoints, and data analysis. In all therapy experiments, mice injected with Namalwa cells or Namalwa/mdr-1 cells were allowed to develop a tumor burden for 7 days before treatments using various drug regimens. Anti-B4–br was diluted appropriately with PBS, pH 7.4, containing 0.1% human serum albumin and injected into the tail veins of tumor-bearing animals. The chemotherapeutic drugs doxorubicin and etoposide were also administered intravenously (IV). Control animals received no treatment, because we have observed previously that treatment with PBS did not alter the survival of tumor-bearing mice. A group receiving anti-B4–br, as well as groups receiving the drugs alone at their maximum tolerated dose (MTD), were included as controls in each combination therapy experiment. The major endpoint of this study was survival, with each treatment group containing 5 to 10 tumor-bearing animals. The survival of all mice was followed, and an MST was calculated for each group. The increase in lifespan (ILS) was calculated by dividing the MST of a treatment group by the MST of the control group, and is expressed as the percent increase over the life span of the control animals. Statistical analysis was performed by the log-rank test and the Wilcoxon test at the 5% significance level.

Assessment of P-glycoprotein expression on Namalwa/mdr-1 tumor cells. Namalwa/mdr-1 cells were recovered from SCID mice bearing late-stage tumors (five mice per group) that either received no treatment or were treated with anti-B4–br (50 or 75 μg/kg/d × 5). A single-cell suspension was prepared by mincing tumors through metal grids followed by Ficol Hypaque gradient centrifugation to remove red blood cells, dead cells, and debris. Cells collected from each untreated mouse on day 20 were stained with the 4E3 and anti-B4 monoclonal antibodies at the time of harvest, and a portion of the cells was cryopreserved to be stained in parallel with cells collected from each anti-B4–br–treated mouse on day 49.

RESULTS

Establishment and genotypic characterization of Namalwa/mdr-1 cell line. The Burkitt’s lymphoma-derived
cell line Namalwa has been extensively used as a target for in vitro cytotoxicity studies with the immunotoxin anti-B4–bR. To determine the cytotoxic effects of anti-B4–bR on cells that are drug-resistant due to overexpression of P-glycoprotein, the recombinant retrovirus expression vector pDOL−mdr was introduced into Namalwa cells by infection (see Materials and Methods). After 3 weeks of selection in medium containing 25 nmol/L or 50 nmol/L vincristine, drug-resistant colonies emerged. No cell growth was observed with noninfected Namalwa cells under these conditions. Proliferating colonies were grown in the presence of 50 nmol/L vincristine for an additional 2 weeks. One colony, designated Namalwa/mdr-1, was then cultured in the absence of drug and used for subsequent studies.

Transmission of the pDOL−mdr vector was assessed by Southern blot analysis (Fig 1). DNA from Namalwa cells that were not infected, from Namalwa cells to which pDOL−mdr plasmid marker DNA was added (11 pg, or approximately one gene copy per diploid cell), and from Namalwa/mdr-1 cells was digested with Kpn I. Restriction fragments that migrated at approximately 3.0 and 5.0 kb are visible in Namalwa DNA containing the pDOL− plasmid marker and in Namalwa/mdr-1 cells but not in noninfected cells, which indicates that vector sequences were transmitted intact. The presence of additional bands migrating above the 5.0-kb marker in all lanes represents endogenous mdr sequences.

Expression of CD19 and P-glycoprotein on Namalwa and Namalwa/mdr-1 cells. The levels of expression of P-glycoprotein on Namalwa and Namalwa/mdr-1 cells were assessed by indirect immunofluorescence staining using the 4E3 monoclonal antibody (Fig 2, right panels). Namalwa cells exhibit a low level of P-glycoprotein, which may reside in a subpopulation. Namalwa/mdr-1 cells express a greatly enhanced level of P-glycoprotein. Both cell lines express similar levels of CD19, as assessed by the binding of anti-B4 (Fig 2, left panels). Thus, transfer and expression of the recombinant provirus pDOL−mdr resulted in surface expression of P-glycoprotein on Namalwa cells and did not alter the expression of CD19.

Sensitivity of cell lines to anti-B4−bR and to chemotherapeutic drugs. Namalwa or Namalwa/mdr-1 were incubated for 3 days in the presence of various concentrations of anti-B4−bR, doxorubicin, etoposide, 4-HC, or cisplatin. Surviving cells were then assayed by adding [3H]dT for 18 hours and measuring the incorporation of radioactivity into the DNA. Cultures containing no cytotoxic agent served as controls, while other cultures were incubated with vincristine, the drug used in selection of resistant clones. The IC50 values (the concentration of toxin that reduces [3H]dT incorporation by 50%) were deduced from killing curves derived from these assays and are summarized in Table 1. Interestingly, Namalwa/mdr-1 cells are about twofold more sensitive to the toxicity of anti-B4−bR than wild-type Namalwa cells, with IC50 values of 4 pmol/L and 8 pmol/L, respectively, for the two different cell lines. Namalwa/mdr-1 cells are sixfold more resistant than Namalwa to doxorubicin and etoposide, as expected, because these two drugs are subject to MDR mediated by P-glycoprotein. However, Namalwa/mdr-1 cells are slightly more sensitive than Namalwa to cisplatin (by a factor of 1.5), while both cell lines show comparable sensitivity to 4-HC. Neither of these drugs is subject to P-glycoprotein–mediated drug resistance. This pattern of cytotoxicity confirms the expression of a functional MDR phenotype in Namalwa/mdr-1 cells. Expression of functional P-glycoprotein was also confirmed by measurements in cellular uptake of radiolabeled daunomycin ([3H]-daunomycin; NEN/DuPont, Boston, MA), which determined that Namalwa/mdr-1 cells accumulated one third as much drug as the parent Namalwa cells (data not shown).

Treatment of Namalwa and Namalwa/mdr-1 cells with combinations of anti-B4−bR and doxorubicin or etoposide in vitro. Each agent was initially tested in combination with a range of anti-B4−bR concentrations from 4 to 20 pmol/L. We then chose concentration ranges for each agent that would elicit a 1.5 to 2.0 log reduction in [3H]dT incorporation with the single agent and that could be analyzed in our isobologram program. Because we obtained steeper kill curves with drugs on the drug-sensitive Namalwa cells compared with Namalwa/mdr-1 cells, we had to use slightly lower doses of anti-B4−bR on Namalwa cells to satisfy the criteria of the assay. Namalwa cells were incubated with anti-B4−bR at concentrations of 8 pmol/L or 16 pmol/L, combined simultaneously in doxorubicin concentrations ranging from 2 to 12 nmol/L. The resultant isobolograms with the calculated envelopes of additivity for these combinations are shown in Fig 3A. They indicate at least additive killing at all tested concentrations of anti-B4−bR and doxorubicin. At the higher anti-B4−bR concentration (16 pmol/L) the killing curve lies slightly outside of the envelope of additivity in the area of supra-additivity.
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The drug-resistant Namalwa/mdr-1 cells were treated with 10 pmol/L or 20 pmol/L anti-B4–bR in the presence of doxorubicin at concentrations ranging from 30 to 90 nmol/L. Isobolograms obtained are shown in Fig 3B and indicate that the entire killing curve is displaced to the left of the envelope of additivity, which demonstrates supra-additive killing at both anti-B4–bR concentrations and for the whole range of doxorubicin concentrations tested. The combination of 20 pmol/L anti-B4–bR with doxorubicin concentrations ranging from 50 to 90 nmol/L produces a cytotoxic effect that is approximately one log greater than additive cell killing.

We used a similar strategy for assessing the effects of etoposide combined with anti-B4–bR. Namalwa cells were incubated with anti-B4–bR at concentrations of 8 pmol/L and 16 pmol/L, with each concentration being combined with etoposide concentrations ranging from 10 nmol/L to 90 nmol/L. Isobolograms obtained are shown in Fig 4A. The killing curves for anti-B4–bR at both 8 pmol/L and 16 pmol/L show supra-additive killing. Namalwa/mdr-1 cells were treated with concentrations of anti-B4–bR of 10 pmol/L and 20 pmol/L, combined with higher concentrations of etoposide (from 50 nmol/L to 400 nmol/L). The resulting isobolograms shown in Fig 4B demonstrate that the combination of 10 pmol/L anti-B4–bR with all tested concentrations of etoposide have an additive killing effect, while the combination of 20 pmol/L anti-B4–bR with etoposide leads to supra-additive killing over the whole concentration range of etoposide.

Optimization of conditions for combination therapy in vivo. Based on our in vitro results with combinations of immunotoxin and a drug on Namalwa and Namalwa/mdr-1 cells, we were encouraged to test combination therapies in a SCID mouse model of these tumors. Initial experiments were performed to determine the MTD of the drugs and to determine the optimal doses and schedules of administration for combinations. It was established that while the MTD for anti-B4–bR given as 5 IV injections on 5 successive days is 75 μg/kg/d × 5 days, these doses proved to be too toxic in this model when given in combination with efficacious doses of the chemotherapeutic drugs. A dose of anti-B4–bR of 50 μg/kg/d × 5 days (days 7 through 11) demonstrated a substantial therapeutic effect when given alone (ILS: 46% v 64% at the MTD),3 while allowing the further administration of other chemotherapeutic drugs. Combination schedules were tested where the dose and schedule of anti-B4–bR was held constant at 50 μg/kg/d × 5 days and doxorubicin was administered simultaneously. However, the amount of drug administered to the mice had to be reduced to 66% of the MTD due to toxicity. The optimal protocol was a sequential combination, administering doxorubicin at a dose of 3 mg/kg/d × 3 every 5 days on days 12, 17, and 22 (the interval between injections of doxorubicin was extended to 5 days for this protocol due to toxicity).

Using the sequential combination schedule outlined above, SCID mice bearing Namalwa or Namalwa/mdr-1 tumors

Table 1. Cytotoxicity of Chemotherapeutic Drugs to Namalwa and Namalwa/mdr-1 Cells

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Anti-B4–bR (pmol/L)</th>
<th>Vincristine (nmol/L)</th>
<th>Doxorubicin (nmol/L)</th>
<th>Etoposide (nmol/L)</th>
<th>Cisplatin (nmol/L)</th>
<th>4-HC (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Namalwa</td>
<td>8</td>
<td>0.75</td>
<td>5.8</td>
<td>40</td>
<td>250</td>
<td>1</td>
</tr>
<tr>
<td>Namalwa/mdr-1</td>
<td>4</td>
<td>32</td>
<td>35</td>
<td>250</td>
<td>170</td>
<td>1</td>
</tr>
</tbody>
</table>

Data are IC₅₀ values. IC₅₀ is defined as the concentration of drug that inhibits [³H]dT incorporation by 50% and was calculated from semilogarithmic plots of drug concentration v logarithm of fraction of [³H]dT incorporation from cytotoxicity assays (see Materials and Methods).
Figure 3. Isobolograms for combinations of anti-B4–br with doxorubicin. Namalwa cells (A) and Namalwa/mdr-1 cells (B) were incubated with the indicated concentrations of anti-B4–br and doxorubicin for 72 hours and then pulsed with [3H]dT for 18 hours. Data are presented as the mean and standard deviation of three experiments, where the cpm incorporated into treated cells is expressed as a fraction of cpm incorporated into control cells. The solid lines with closed circles represent the killing curves for doxorubicin alone, solid lines with open triangles represent killing curves for anti-B4–br combined with doxorubicin, and the broken lines represent the boundaries of the envelopes of additivity for the combination treatments.

Figure 4. Isobolograms for combinations of anti-B4–br with etoposide. Namalwa cells (A) and Namalwa/mdr-1 cells (B) were exposed to the indicated concentrations of anti-B4–br combined with etoposide for 72 hours and then pulsed with [3H]dT for 18 hours. The data are presented as the mean and standard deviation cpm calculated as outlined for Fig 3. The solid lines with closed circles represent killing curves for etoposide alone, solid lines with open triangles represent killing curves for anti-B4–br combined with etoposide, and the broken lines represent the boundaries of the envelopes of additivity for the combination exposures.

Treatment of SCID mice bearing Namalwa/mdr-1 tumors with a sequential combination of anti-B4–br and doxorubicin effected an ILS of 129% (P < .0005 v anti-B4–br alone; P < .0005 v control, drug alone, or anti-B4–br alone).

Treatment of SCID mice bearing Namalwa/mdr-1 tumors with a sequential combination of anti-B4–br and doxorubicin effected an ILS of 129% (P < .0005 v anti-B4–br alone; P < .0005 v control, drug alone, or anti-B4–br alone), which was a dramatic improvement in therapeutic efficacy when compared with anti-B4–br (ILS, 46%) or doxorubicin (no therapeutic efficacy) used as single agent treatments (Table 2, experiment B; survival curves in Fig 5B). Similar results were obtained with a combination of anti-B4–br and etoposide (Table 2, experiment D; Fig 5D). Etoposide alone showed no significant therapeutic efficacy compared with untreated controls, while the anti-B4–br/etoposide combination gave an ILS of 115% (P < .0005 v control or drug alone), which was
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Table 2. Summary of Doses and Treatment Protocols for Anti-B4-bR and Drug Combinations

<table>
<thead>
<tr>
<th>Treatment Protocol</th>
<th>MST (d)</th>
<th>ILS (%)</th>
<th>Pt</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment A: Namalwa</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Untreated control</td>
<td>23</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>2. Anti-B4-bR (50 μg/kg/d x 5 d)</td>
<td>33</td>
<td>43</td>
<td>&lt;.001 v 1</td>
</tr>
<tr>
<td>3. Doxorubicin (3 mg/kg/d x 3 every 4 d)</td>
<td>29</td>
<td>26</td>
<td>&lt;.05 v 1</td>
</tr>
<tr>
<td>4. Anti-B4-bR (50 μg/kg/d x 5 d) + doxorubicin (3 mg/kg/d x 3 every 5 d)</td>
<td>48</td>
<td>109</td>
<td>&lt;.0005 v 1, 3 &lt;.001 v 2</td>
</tr>
<tr>
<td><strong>Experiment B: Namalwa/mdr-1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Untreated control</td>
<td>24</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>2. Anti-B4-bR (50 μg/kg/d x 5 d)</td>
<td>35</td>
<td>46</td>
<td>&lt;.001 v 1</td>
</tr>
<tr>
<td>3. Doxorubicin (3 mg/kg/d x 3 every 4 d)</td>
<td>25</td>
<td>4</td>
<td>NS</td>
</tr>
<tr>
<td>4. Anti-B4-bR (50 μg/kg/d x 5 d) + doxorubicin (3 mg/kg/d x 3 every 5 d)</td>
<td>55</td>
<td>129</td>
<td>&lt;.0005 v 1, 3</td>
</tr>
<tr>
<td><strong>Experiment C: Namalwa</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Untreated control</td>
<td>25</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>2. Anti-B4-bR (50 μg/kg/d x 5 d)</td>
<td>36</td>
<td>44</td>
<td>&lt;.001 v 1</td>
</tr>
<tr>
<td>3. VP-16 (15 mg/kg/d x 3 every 2 d)</td>
<td>33</td>
<td>32</td>
<td>&lt;.01 v 1</td>
</tr>
<tr>
<td>4. Anti-B4-bR (50 μg/kg/d x 5 d) + VP-16 (15 mg/kg/d x 3 every 4 d)</td>
<td>57</td>
<td>128</td>
<td>&lt;.0005 v 1, 2, 3</td>
</tr>
<tr>
<td><strong>Experiment D: Namalwa/mdr-1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Untreated control</td>
<td>27</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>2. Anti-B4-bR (50 μg/kg/d x 5 d)</td>
<td>42</td>
<td>56</td>
<td>&lt;.001 v 1</td>
</tr>
<tr>
<td>3. VP-16 (15 mg/kg/d x 3 every 2 d)</td>
<td>28</td>
<td>4</td>
<td>NS</td>
</tr>
<tr>
<td>4. Anti-B4-bR (50 μg/kg/d x 5 d) + VP-16 (15 mg/kg/d x 3 every 4 d)</td>
<td>58</td>
<td>115</td>
<td>&lt;.0005 v 1, 3</td>
</tr>
</tbody>
</table>
| **Abbreviations:** VP-16, etoposide; NS, not significant. **Treatment started on day 7 after inoculation of mice with 4 x 10⁶ tumor cells. **Treatment with drugs started on day 12 after treatment with anti-B4-bR on days 7-11. **Analyzed by the Wilcoxon test at the 5% significance level. **Significantly superior to treatment with anti-B4-bR alone (P < .001). Notably, the therapeutic efficacy of both anti-B4-bR/drug combinations on both the drug-sensitive Namalwa tumor and the drug-resistant Namalwa/mdr-1 tumor were similar, suggesting that anti-B4-bR, in effect, completely reverses the MDR phenotype.

Effect of anti-B4-bR treatment on levels of P-glycoprotein expression in Namalwa/mdr-1 populations in vitro and in vivo. The synergy between anti-B4-bR and drugs could be brought about at the cellular level by an effect of the immunotoxin on drug accumulation or, alternatively, by reducing the level of expression of P-glycoprotein in the cell population, thereby increasing the susceptibility of the cells to drugs. We were unable to show a detectable change in the rate of uptake or efflux of [³H] daunomycin in Namalwa/mdr-1 cells exposed to anti-B4-bR in vitro for different periods of time. However, these assays might not have been sensitive enough to detect small changes in P-glycoprotein function. We, therefore, measured the cell surface levels of P-glycoprotein in the Namalwa/mdr-1 cell population after treatment with anti-B4-bR both in vitro and in vivo. When Namalwa/mdr-1 cells were treated with anti-B4-bR at concentrations of 16 pmol/L, 50 pmol/L, and 250 pmol/L for 72 hours in vitro, indirect immunofluorescence measurements were suggestive of a slight decrease in the level of P-glycoprotein (data not shown). The effects in vivo were more pronounced. Figure 6 shows the expression of P-glycoprotein on the surface of Namalwa/mdr-1 cells recovered from tumor-bearing mice that had been treated with anti-B4-bR (75 μg/kg/d x 5 days, days 7 through 11), compared with cells harvested from untreated controls on day 21. After the treatment with immunotoxin there is a clear emergence of a subpopulation expressing lower levels of P-glycoprotein. In contrast, Namalwa/mdr-1 cells harvested from untreated mice retain high levels of P-glycoprotein expression and show a similar distribution of expression as cells maintained in vitro (compare Fig 6 with Fig 2). The average values for relative mean fluorescence decreased from 486 to 267 (arbitrary units). Namalwa cells from both treated and untreated mice retain high homogenous levels of CD19 expression. Similar data were obtained with each mouse in the treatment group and with Namalwa/mdr-1 cells harvested from SCID mice treated as above with 50 μg/kg/d x 5 days of anti-B4-bR.

DISCUSSION

Efforts at improving the efficacy of combination drug therapy in the treatment of B-cell malignancies have focused on using more intensive treatment, with some protocols containing as many as 6 to 8 different drugs. Recent reports, however, indicate that the use of these second and third generation regimens do not reveal any advantages in terms of disease-free survival compared with the first generation, four-drug regimen CHOP.27,28 The newer drug protocols do have disadvantages in terms of toxicity and cost. These results suggest that existing drug combinations may have reached their limit in terms of dose and toxicity, and new approaches are needed to overcome the problem of resistant tumor cells.

In this report, we establish a rationale for combining the immunotoxin anti-B4-bR with chemotherapy by showing that this immunotoxin enhances the cell killing achieved both in vitro and in vivo by doxorubicin and etoposide on Namalwa cells and the multidrug-resistant Namalwa/mdr-1 cells. Anti-B4-bR can be combined with doxorubicin and etoposide to give additive and synergistic effects, respectively, on Namalwa cells. In addition, anti-B4-bR combined with doxorubicin or etoposide effects supra-additive killing of Namalwa/mdr-1 cells in vitro. The synergy shown between anti-B4-bR and drugs in vitro on the Namalwa/mdr-1 tumor provides an explanation for the dramatic results in an in vivo survival model of the drug-resistant tumor. Neither doxorubicin nor etoposide alone was effective in treating the drug-resistant tumor, but treatment with anti-B4-bR essentially reversed the resistance of the tumor to both drugs. When the increase in life-span obtained with anti-B4-bR and drugs on the drug-sensitive Namalwa tumor is compared with the effect on the drug-resistant tumor, it appears that
the reversal of the MDR phenotype by the immunotoxin is complete in Namalwa/mdr-1 cells.

In vitro cytotoxicity assays consistently showed that Namalwa/mdr-1 cells were about twofold more sensitive to anti-B4-Br than the parent Namalwa cells. Similar results were obtained with a multidrug-resistant variant of the HL-60 cell line that had been selected by adaptive growth in vincristine-containing medium. This cell line was found to be threefold more sensitive than the parent cells to the immunotoxin anti-My9-blocked ricin directed to the CD33 antigen (R.O'C. and Y.L., unpublished observations, 1994). We speculate that this small difference in sensitivity could contribute to synergism between anti-B4-Br and drugs on Namalwa/mdr-1 cells by selectively eliminating the higher P-glycoprotein-expressing cells with the immunotoxin, leaving a population of cells with lower levels of P-glycoprotein expression, which would be expected to be more sensitive to drugs. In the SCID mouse model, after treating Namalwa/mdr-1 cells with anti-B4-Br and waiting until residual tumor cells grew back, we could see the outgrowth of cells expressing lower levels of P-glycoprotein. The same phenomenon could explain the synergism detected with anti-B4-Br and etoposide on Namalwa cells, because Namalwa cells do express P-glycoprotein, albeit at lower levels than Namalwa/mdr-1 cells. We are now designing experiments to further investigate the unexpected observation of a possible correlation between P-glycoprotein expression and the sensitivity of cells to immunotoxins containing blocked ricin.

These findings have important clinical applications. After remission induction therapy, patients are often left with drug-resistant minimal residual disease in which one mechanism of resistance is the overexpression of P-glycoprotein. The
results reported here suggest that blocked ricin-containing immunoconjugates are more effective against drug-resistant cells expressing P-glycoprotein and may be suitable agents to treat at the minimal residual disease stage, with the potential for prolonging disease-free survival or possibly effecting cures. In another scenario, blocked ricin-containing conjugates could be added to the initial chemotherapy regimen, thereby killing drug-resistant cells at their generation and contributing to a deeper remission. The improved therapeutic efficacy of the combination treatments in the tumor-bearing SCID mouse model provides experimental support for the clinical observation that treatment with anti-B4–br may appeared to sensitize some patients to further chemotherapy treatment (L. Nadler, M. Grossbard, A. Levine, personal communication, 1994).

A number of agents such as verapamil, FK506, quinidine, and immunotoxins directed specifically against P-glycoprotein and immunotoxins have been shown in vitro to reverse drug-resistance or to kill tumor cells that express high levels of P-glycoprotein. Verapamil and cyclosporin A have also been tested in clinical trials. However, the effectiveness of verapamil in clinical trials was dose-limited due to cardiotoxicity, and although cyclosporin A could be administered to reach effective levels without toxicity, it was demonstrated to alter the pharmacokinetics of the drugs with which it was administered. One of the problems associated with using the above agents for the reversal of multidrug resistance is their lack of specificity; such drugs could potentially sensitize nontumor cells to the action of drugs. Likewise, an immunotoxin directed to P-glycoprotein would not only target tumor cells expressing high levels of P-glycoprotein but might also be directed to normal kidney, liver, and colon. An immunotoxin targeted to CD19 on drug-resistant B-cell tumors could offer an advantage in terms of causing fewer nonspecific toxic effects to surrounding tissues by virtue of its specificity.

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Anti-B4-blocked ricin synergizes with doxorubicin and etoposide on multidrug-resistant and drug-sensitive tumors

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