B-Cell Restricted Saporin Immunotoxins: Activity Against B-Cell Lines and Chronic Lymphocytic Leukemia Cells

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ImmunoToxins ARE a novel class of reagents that combine the high specificity of monoclonal antibodies with the potent killing activity of ribosome-inactivating proteins. The therapeutic potential of monoclonal antibody-toxin conjugates is actively being investigated both in vitro and in vivo. B-cell neoplasias (non-T-cell acute and chronic lymphocytic leukemias, non-Hodgkin’s lymphomas) are particularly suitable for such a study as the surface-antigen profile of the B cell is well studied, and several B cell-restricted determinants have been identified. In addition, the availability of circulating tumor cells offers the opportunity to study the effect of immunotoxin treatment on their direct target. We and others have studied the anti-tumor activity of monoclonal antibody-ricin A-chain conjugates on neoplastic B-cell lines. Though effective, these immunotoxins require long incubation times (12-24 hours) and the presence of an enhancer molecule in the incubation medium. The ricin A chain may not be the toxic moiety of choice for in vivo studies. Due to its glycolipidation pattern, the ricin A chain is rapidly cleared in vivo by the reticuloendothelial system. The bond between the ricin A chain and the antibody is labile in vivo, accounting for the brief half-life of intact conjugate. In addition, once it has entered the cell, it is easily inactivated by intracellular proteases. Ricin is the prototype of type 2 ribosome-inactivating proteins, ie, toxins composed of a binding moiety (B chain) disulphide linked to a toxic polypeptide (A chain). Other recently described type 1 ribosome-inactivating proteins, ie, toxins devoid of cell-binding activity such as saporin (SAP) and pokeweed antiviral protein (PAP), have been conjugated to anti-B monoclonal antibodies. Uckun et al. have reported that a PAP-containing anti-CD19 immunotoxin (B43-PAP) can eliminate three to four logs of fresh B-lineage acute lymphoblastic leukemia cells under conditions potentially achievable in vivo. We have recently described an anti-CD5 monoclonal antibody-SAP conjugate, referred to as TEC IgM-SAP, which is suitable for ex vivo treatment of the bone marrow. TEC IgM-SAP efficiently kills B-cell lines in a short incubation time in the presence of unseparated bone marrow. In addition, we have reported that a CD5-specific SAP immunotoxin, referred to as OKT1-SAP, is a potent and specific anti-T-cell reagent. Once injected into cynomolgus monkeys, it remains immunologically intact in the blood stream and retains its biologic activity up to two days after administration.

Given these encouraging results, we have looked for B cell-restricted determinants that can be useful targets for immunotoxin activity. The 95 kD antigen, defined by the cluster of differentiation 19 (CD19) according to Nadler, is the broadest lineage-specific surface marker for B cells. It is expressed on virtually all normal and neoplastic B cells, with the exclusion of plasma cells. The 130- to 140-kD glycoprotein heterodimer defined by the CD22 is expressed on the cell surface of a subset of B-cell neoplasias and is B cell-restricted. HD37 is a monoclonal antibody specific for CD19. HD39 and HD6 recognize two different epitopes on the same glycoprotein antigen CD22. We now describe our results with immunotoxins obtained through the conjugation of these B cell-restricted antibodies with SAP. The resulting immunotoxins (HD37-SAP, HD39-SAP, and HD6-SAP) are both efficient and specific reagents against B-cell lines, as they can eliminate more than two logs of clonogenic lymphoma cells after a two-hour incubation in the absence of potentiators. Recently, it has been demonstrated that fresh B-chronic lymphocytic leukemia (B-CLL) cells can be induced to proliferate by a combination of anti-CR1 and anti-T-cell reagent.
antibody and B-cell growth factor, properly sequenced. The availability of this new technique prompted us to determine the efficacy of these immunotoxins on fresh B-CLL cells. We demonstrate that anti-CD19 and CD22 immunotoxins inhibit the proliferation of B-CLL cells under conditions achievable in vivo without the need of potentiators.

MATERIALS AND METHODS

Cell lines. The B-cell lines Bjab I, Namalwa, CA46, JD40, and the T-cell line CEM were used in this study. Cell lines were a gift from Dr R. C. Bast, Duke University, Durham, NC. Cells were growth in RPMI 1640 medium with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 mcg/mL streptomycin, at 37°C with 5% CO₂. Cells were split 18–24 hours before experiments to ensure optimal growth.

Monoclonal antibodies. J5 is an IgG2a monoclonal antibody specific for the common acute lymphoblastic leukemia antigen (CALLA), defined by CD10 (Coulter Immunology, Hialeah, FL). B1 is directed against CD20, a 35-kD surface determinant present on most B cells (Coulter Immunology). OKT11 recognizes CD22, a T cell–specific antigen (Ortho System, Raritan, NJ). OKT1 detects a 65-kD antigen (CD5), which is present on normal and neoplastic T cells as well as on most B-CLL cells (Ortho System). OKT3 is specific for the T-cell antigen receptor CD3 (Ortho System). Mo1 and My7 are two monoclonal antibodies that detect two different antigens restricted to myeloid differentiation (Coulter Immunology).

HD37 is a murine IgG1 specific for CD19, a 95-kD B cell–restricted antigen. HD39 and HD6 are murine IgG1 antibodies that recognize two different epitopes on the same CD22 molecule, a 130- to 140-kD glycoprotein heterodimer which is B cell–restricted. For indirect immunofluorescence studies, cells were incubated 30 minutes on ice with the relevant antibody, washed three times, and incubated 30 minutes in ice with a fluorescein-conjugated goat anti-mouse antiserum (Technogenetics, S. Mauro Toninese, Italy). The number of positive cells and relative fluorescence intensity were evaluated by a FACSTAR cell sorter (Becton Dickinson, CA). For conjugation to SAP, antibodies were purified by ammonium sulphate precipitation and fast protein liquid chromatography over a mono-S column (Pharmacia Fine Chemicals, Uppsala, Sweden).

Immunotoxin synthesis. SAP is a type I ribosome-inactivating protein of 30,000 MW, extracted from the seeds of the plant Saponaria officinalis. SAP was prepared as previously described and was coupled to HD37, HD39, and HD6 monoclonal antibodies according to published techniques. Briefly, the immunotoxin was synthesized with the cross-linker N-succinylimidyl-3-(2-pyridyl)propionate (SPDP, Pharmacia, Uppsala, Sweden). Derivatization of the antibody and SAP was carried out according to the manufacturer’s instructions. The average number of SAP molecules per molecule of antibody was 1.5 (range 1–2). The immunotoxin was purified by ion-exchange chromatography using the difference in the isoelectric points between SAP (10.5) and the antibody (<7.6). Reaction mixtures were passed over Sephadex G-25 (Pharmacia) equilibrated in 25 mM sodium citrate, pH 5. The fractions of the excluded volume were applied to an FPLC mono-S column equilibrated with the same buffer and eluted with a gradient of 0–0.3 M NaCl. Fractions were analyzed by sodium dodecyl sulfate (SDS)-gel electrophoresis, and fractions containing conjugated antibody were pooled and dialyzed versus Dulbecco’s phosphate buffered saline (PBS).

Immunotoxin treatment of B-cell lines. Cells were incubated in RPMI 1640-10% fetal bovine serum (FBS) at 10⁶ cells/mL, and tested immunotoxin was added at the desired final concentration as a 10X concentrate. After 15 minutes on ice and 120 minutes at 37°C, cells were washed four times and the effect of immunotoxin treatment was evaluated by the following assays.

(a) Inhibition of protein synthesis. Cells (10⁶/mL) were seeded in 96 U-bottomed well plates and pulsed with 1 μCi/well tritiated leucine (³H-Leu) for three hours at 37°C in the presence of 5% CO₂, 95% humidified air. After trichloroacetic acid (TCA) precipitation, cell proteins were collected on glass fiber filters and incorporated radioactivity was assessed by a standard scintillation counting technique.

(b) Inhibition of DNA synthesis. 10⁶ cells in 100 μL RPMI 1640-10% FBS were seeded in 96 U-bottomed well plates (Costar, Cambridge, MA) and pulsed for three hours with ³H-thymidine, 1 μCi/well, at 37°C in the presence of 5% CO₂. All experiments were done in triplicate. Cells were harvested by a Skatron apparatus, and incorporation was quantified by scintillation counting. The cytotoxicity was evaluated as a percentage of untreated control counts.

(c) Clonogenic assay. The survival of clonogenic cells was evaluated by a limiting dilution assay, as already described. Briefly, 10⁶ cells were resuspended in 1 mL RPMI 1640-10% FBS and incubated with the immunotoxin at the desired concentration for 15 minutes on ice, and for two hours at 37°C with occasional agitation. Cells were washed three times and resuspended at 10⁶/mL in 1 mL RPMI-10% FBS. Ten serial five-fold dilutions were made in the same medium. Six aliquots (100 μL) from each dilution were plated in 96-flat-bottomed well plates (Costar), seeded with 100 μL of irradiated (5,000 rad) mononuclear bone marrow cells as a feeder layer. Growth of lymphoma colonies was scored after 12–14 days of incubation at 37°C in 5% CO₂, 95% humidified air. The number of clonogenic units was calculated using a Spearman-Karber estimator.

Immunotoxin treatment of B-CLL cells. Peripheral blood cells were obtained from 31 consecutive patients with B-CLL. The clinical features of the patients (age, sex, white blood cell count [WBC] and absolute lymphocyte count, Rai stage, previous and present therapy) were recorded. Mononuclear cells were separated from peripheral blood by Ficol-Hyphaque density gradient and washed twice with Hank’s balanced salt solution (HBSS) with 5% FBS. The cell-surface phenotype was determined by indirect immunofluorescence with anti-CD3, CD5, CD11b, CD19, CD20, and slg monoclonal antibodies. When CD3-positive cells were more than 5%, T cells were eliminated by AET-SRBC rosetting. B-CLL cells were incubated in RPMI 1640-10% FBS at 2 × 10⁶ cells/mL in 96 flat-bottomed well plates (Costar), and tested immunotoxin was added at 50 nM final concentration. After 15 minutes on ice and two hours at 37°C, the cells were washed and the effect of the treatment was evaluated as described below. In some cases, immunotoxin was added to fresh, unseparated blood at 50 nM final concentration at 37°C. After two hours of incubation, mononuclear cells were obtained by Ficol-Hyphaque separation, and the effect of the treatment was evaluated as described below.

B-CLL cell proliferation inhibition assay. B-CLL cells were induced to proliferate according to a method already described, with modifications. Briefly, CB04 is a monoclonal antibody that reacts with CR1 (the receptor for the C3b complement component), an antigen of 220 kD defined by the cluster of differentiation CD35. CB04 purified from ascites was dialyzed, filtered, and covalently linked to activated Sepharose CL-4B (Pharmacia) by the cyanogen bromide technique (CB04-S). B-CLL cells were resuspended in RPMI 1640-10% FBS at 2 × 10⁶ cells/mL and cultured in the presence of CB04S at 37°C in humidified atmosphere with 5% CO₂ in 96 U-bottomed well plates. After 72 hours, low-molecular-weight XB-cell growth factor (BCGF, Cellular Products, Buffalo, NY) was added to the culture at the final concentration of 10% vol/vol. This factor, obtained from mitogen-stimulated peripheral blood lympho-
cytes, supports the short-term proliferation of activated human B but not T cells. After additional 72 hours, 3H-thymidine (1 μCi/well) was added to the culture, and incubation was continued for 18 hours. The cells were subsequently harvested onto glass fiber filters by a Skatron harvester and counted in a liquid scintillation counter. All experiments were done in triplicate. Stimulation index is defined as ratio of 3H-TdR incorporation in stimulated cells to unstimulated medium-treated cells. The inhibition of B-CLL cell proliferation was evaluated as a percentage of 3H-TdR incorporation, according to the following formula: stimulated immunotoxin-treated (cpm) – unstimulated medium-treated (cpm) / stimulated medium-treated (cpm) – unstimulated medium treated (cpm).

RESULTS

Reactivity of monoclonal antibodies. CD19 and CD22 antigens are expressed on ≥85% cells from most B cell lines. Namalwa cells uniformly express CD19, but only about 50% of cells express CD22 antigen. The T cell line CEM (a leukemia cell line) does not express CD19 and CD22 (Table 1).

Immunotoxin treatment of cell lines. Inhibition of DNA synthesis. Cells were incubated with increasing molar concentrations of HD37-SAP, HD39-SAP, and HD6-SAP for two hours at 37°C, and 3H-TdR incorporation was compared to untreated controls. As shown in Fig 1, the DNA synthesis of all tested cell lines was inhibited in a dose-related manner. Immunotoxins were more effective against Bjab 113, CA46, and JD40 cell lines whereas the growth of Namalwa was only marginally affected. Individual cell lines differed in their sensitivity to immunotoxins. The DNA synthesis-inhibition activity was not correlated to the expression of the relevant antigens on the cell surface, in that all cell lines, with the partial exception of Namalwa, were highly reactive with anti-CD19 and CD22 antibodies. A more effective DNA synthesis inhibition was obtained at the same immunotoxin doses by incubation of the cells for 16 hours (data not shown). The 3H-TdR incorporation by CEM cells was not affected even at higher immunotoxin doses (Fig 1) or longer incubation times (data not shown).

Inhibition of protein synthesis. Since ribosome-inactivating proteins inhibit protein synthesis of target cells, we studied the inhibition of 3H-leucine incorporation in two target cell lines, Namalwa and Bjab 113, which have different sensitivity to the activity of immunotoxins, as shown by the above DNA synthesis-inhibition studies.

Table 1. Cell-Surface Phenotype of Cell Lines

<table>
<thead>
<tr>
<th>Antibody</th>
<th>JD40</th>
<th>CA46</th>
<th>Bjab 113</th>
<th>Namalwa</th>
<th>CEM</th>
</tr>
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<tbody>
<tr>
<td>J5</td>
<td>92</td>
<td>84</td>
<td>80</td>
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<tr>
<td>B1</td>
<td>91</td>
<td>63</td>
<td>85</td>
<td>5</td>
<td>11</td>
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<tr>
<td>HD37</td>
<td>92</td>
<td>95</td>
<td>89</td>
<td>96</td>
<td>3</td>
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<td>89</td>
<td>95</td>
<td>86</td>
<td>50</td>
<td>2</td>
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<tr>
<td>HD6</td>
<td>91</td>
<td>94</td>
<td>87</td>
<td>47</td>
<td>3</td>
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<td>7</td>
</tr>
<tr>
<td>My7</td>
<td>0</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>0</td>
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</table>

10⁶ cells from each cell line were incubated with monoclonal antibody, washed, and incubated with FITC-conjugated goat anti-mouse antiserum. Percentage of positive cells was determined by FACSTAR fluorescence-activated cell sorter.

As shown in Table 2, after a two-hour incubation at 37°C, the ID50 of HD37-SAP was 2.8 x 10⁻⁹ M for Namalwa and 4.9 x 10⁻¹¹ M for Bjab 113. The ID50 of HD39-SAP were 4.9 x 10⁻¹⁰ M and 3.1 x 10⁻¹⁰ M for Namalwa and Bjab 113, respectively, and the ID50 of HD6-SAP were 6.6 x 10⁻⁹ M and 1.2 x 10⁻¹¹ M for Namalwa and Bjab 113, respectively. Bjab 113 protein synthesis was 10- to 50-fold more sensitive to immunotoxin activity than Namalwa, which showed a relative resistance to the anti-CD19 immu-
notoxin. After a 16-hour incubation, the ID50s of all three immunotoxins for Bjab 113 decreased by approximately one log. The ID50 of free SAP was 1,000- to 2,000-fold higher than the ID50 of the conjugated toxin after a two-hour incubation (1.6 x 10^-4 M for Bjab 113 and 1.1 x 10^-6 M for Namalwa).

**Inhibition of clonogenic cell growth.** The endpoint of in vivo immunotoxin use in the treatment of B-cell malignancies is the elimination of the clonogenic tumor cells from the patient. A limiting dilution assay was employed to define the clonogenic survival of neoplastic cells after treatment with immunotoxins. Cells from Namalwa, Bjab 113, and CEM lines were incubated for two hours with different concentrations of HD37-SAP immunotoxin, and the percentage of surviving cells was scored after 14 days in culture (Fig 2). More than two logs (99.5%) of clonogenic Bjab 113 cells were eliminated by this short-term incubation with 100 nM immunotoxin. Less effective (95%) cell elimination was achieved using the same conditions on Namalwa. CEM cell growth was unaffected by the same treatment even at the higher concentrations. The activity of HD39-SAP and HD6-SAP was evaluated on Bjab 113 and CEM cells by a two-hour incubation clonogenic assay (Fig 2). Both immunotoxins were highly efficient in tumor cell elimination. HD39-SAP achieved 99.5% and HD6-SAP 99.2% clonogenic cell elimination. In a clonogenic assay, all three immunotoxins appear equally effective in Bjab 113 tumor cell killing. As predicted by DNA and protein synthesis inhibition assays, Namalwa was less sensitive to immunotoxin action than Bjab 113. CEM cells were not killed by anti-CD22 conjugates.

**Immunotoxin specificity.** As detailed above, CEM cells that do not express target antigens were not killed by immunotoxin treatment. In addition, 50 nM antibody plus 50 nM free saporin did not affect the ³H-TdR uptake of Bjab 113 and Namalwa cells, as compared to 50 nM immunotoxins (Table 3). Similarly, the killing of Bjab 113 cells by the immunotoxins was completely inhibited when cells were preincubated with a 100-fold excess of unconjugated relevant antibody (data not shown).

**Immunotoxin treatment of B-CLL cells. Patients.** Twenty-four consecutive patients with B-CLL were investigated. Clinical features of these patients were heterogeneous with regard to Rai stage, percentage of B-CLL circulating peripheral blood cells, and previous and concurrent therapy. Blood specimens were drawn at least three weeks after the last course of therapy. The reactivity of the CLL cells with anti-CD3, CD5, CD11b, CD19, CD20, and

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**Table 2. Protein Synthesis Inhibition of B Cell Lines by Anti-B Immunotoxins**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>ID50 (M)</th>
<th>2 hr</th>
<th>16 hr</th>
<th>2 hr</th>
<th>16 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Namalwa</td>
<td>HD37-SAP</td>
<td>2.8 x 10^-9</td>
<td>ND</td>
<td>4.9 x 10^-11</td>
<td>2.6 x 10^-12</td>
</tr>
<tr>
<td></td>
<td>HD39-SAP</td>
<td>4.9 x 10^-10</td>
<td>ND</td>
<td>3.1 x 10^-11</td>
<td>3.7 x 10^-12</td>
</tr>
<tr>
<td></td>
<td>HD6-SAP</td>
<td>6.6 x 10^-10</td>
<td>ND</td>
<td>1.2 x 10^-11</td>
<td>1.4 x 10^-12</td>
</tr>
<tr>
<td></td>
<td>SAP</td>
<td>1.1 x 10^-8</td>
<td>1.2 x 10^-8</td>
<td>1.6 x 10^-8</td>
<td>1.9 x 10^-9</td>
</tr>
<tr>
<td>Bjab 113</td>
<td>HD37-SAP</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>HD39-SAP</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td></td>
<td>HD6-SAP</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>SAP</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*a^a cells from Namalwa and Bjab 113 cell lines were incubated in triplicate with 10^-12 M to 10^-7 M HD37-SAP, HD39-SAP, and HD6-SAP. 3H-Leu incorporation inhibition in treated samples was determined as described in Materials and Methods. The ID50 (concentration inhibiting 50% protein synthesis) is reported for each immunotoxin.

**Fig 2.** Clonogenic survival of immunotoxin-treated cells. 10^6 cells from Namalwa, Bjab 113, and CEM cell lines were incubated for two hours with 0.01–100 nM HD37-SAP, HD39-SAP, and HD6-SAP. After washings, cells were plated in a clonogenic limiting dilution assay (see Materials and Methods for details). The percentage of clonogenic surviving cells was calculated by comparison to untreated controls.
CD22 was determined. All cells were uniformly CD19 and CD20 positive, while heterogeneity was observed in the expression of CD22 antigen. Most of our B-CLL cases, however, were CD22 positive at low antigen density.

**Immunotoxin treatment.** Mononuclear peripheral blood B-CLL cells were separated on Ficoll-Hypaque density gradient, treated with 50 nM immunotoxin for two hours at 37°C, washed, and induced to proliferate by the sequential addition of CB04-S and BCGF. After 72 hours, ³H-TdR was added to the wells. Cytotoxicity was evaluated by comparison with the untreated controls (see Materials and Methods). Most samples (87.5%) showed a significant increase in ³H-TdR incorporation as compared to the unstimulated control. Wide variations in ³H-TdR incorporation were observed, but in most evaluable cases (76%), the stimulation index was >10. Treatment with 50 nM HD37-SAP, HD39-SAP, and HD6-SAP actively inhibited growth of B-CLL cells. Mean HD37-SAP inhibitory activity was 83.5% (range 54-99), and >50% in all cases (Table 4). Evaluation of the activity of anti-CD22 immunotoxins was limited to 20 cases. HD39-SAP and HD6-SAP were also actively cytotoxic (mean 73.4%, range 26-99, and 74.1%, range 34-99 respectively) (Table 5). The percentage of inhibition of DNA synthesis by HD37-SAP was weakly correlated to the percentage of CD19-positive cells (R = 0.38), and this correlation was weaker in the case of HD39-SAP (R = 0.27) and HD6-SAP (R = 0.30). Immunotoxin activity was also unrelated to the stage of the disease and to the status of therapy of the patient. In some patients, HD39-SAP and HD6-SAP failed to achieve more than 50% DNA synthesis inhibition. However, in one of these patients (UPN 74-115), the expression of CD22 antigen was virtually absent (Table 5). In all cases of HD37-SAP treatment, more than 50% ³H-TdR incorporation inhibition was observed.

**Specificity.** Normal B cell proliferation, induced by stimulation with anti-Ig antiserum plus BCGF, can be inhibited by pre-incubation with HD37 antibody. We tested the effect of unconjugated antibodies and free saporin on B-CLL cells. While HD39 and HD6 did not exert any

### Table 3. Effect of Unconjugated Antibodies and Free Saporin on DNA Synthesis of B Cell Lines

<table>
<thead>
<tr>
<th>Treatment</th>
<th>3H-TdR Uptake (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>172,960 65,350</td>
</tr>
<tr>
<td>HD37 Ab + SAP</td>
<td>187,320 (100)*</td>
</tr>
<tr>
<td>HD37-SAP</td>
<td>7,451 (4.3)</td>
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<tr>
<td>HD39 Ab + SAP</td>
<td>143,760 (83.1)</td>
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<tr>
<td>HD39-SAP</td>
<td>1,215 (0.7)</td>
</tr>
<tr>
<td>HD6 Ab + SAP</td>
<td>211,720 (100)</td>
</tr>
<tr>
<td>HD6-SAP</td>
<td>1,463 (0.8)</td>
</tr>
<tr>
<td>Free SAP</td>
<td>118,100 (68.2)</td>
</tr>
</tbody>
</table>

Cells were treated with 50 nM immunotoxin or 50 nM antibody plus 50 nM saporin for two hours. ³H-TdR incorporation inhibition in treated samples was determined according to Materials and Methods.

* ( ) = percent of control.
effect on the $^3$H-TdR incorporation of treated cells, in some experiments HD37 antibody showed a partial inhibitory activity on leukemic cell proliferation. Incubation of cells from patient 86-370 with 50 nM HD37-SAP produced 95% DNA synthesis inhibition, and incubation with 50 nM unconjugated HD37 resulted in 48% inhibition. Fifty nM free SAP did not inhibit DNA synthesis (89% DNA synthesis of untreated control). DNA synthesis in cells from patient 71-392 dropped to 8% of control after immunotoxin treatment and to 60% after antibody treatment. SAP treatment did not affect $^3$H-TdR incorporation (98% of control). Neither incubation with 50 nM HD39 nor HD6 caused inhibition of DNA synthesis in treated cells. Preincubation of B-CLL cells with 100-fold molar concentration of unconjugated CD22 antibodies ($10^{-6}$ M) prevented most of the cytotoxic effect of the relevant immunotoxin (Fig 3).

Dose-dependent activity of the immunotoxins on B-CLL cells. All B-CLL cell treatments were done at 50 nM immunotoxin concentration, a concentration achievable in vivo after administration of nontoxic doses of immunotoxin.\(^{12}\) In two cases, a dose-response curve of DNA synthesis inhibition was obtained, with an immunotoxin concentration range from $10^{-10}$ M to $10^{-7}$ M. A representative experiment is shown in Fig 4. The ID50s of the three immunotoxins are reported in Table 6.

Activity of immunotoxins on whole blood. In some cases, the immunotoxins were added at 50 nM final concentration in 1 mL of fresh unseparated CLL blood at 37°C, under conditions close to the clinical situation. After treatment, mononuclear cells were separated by Ficoll/Hyphaque density gradient, and immunotoxin activity was evaluated by inhibition of $^3$H-TdR incorporation as described above. In six out of seven cases, a good inhibitory activity was obtained by treatment of fresh unseparated blood (Table 7). A representative experiment in which immunotoxin activity
was evaluated on whole blood and Ficoll-Hypaque separated mononuclear cells from the same patient (UPN 87-656) failed to show major differences in the immunotoxin inhibitory activity (Fig 5). All three immunotoxins are active on target cells in the presence of plasma and red blood cells/granulocytes.

**DISCUSSION**

In this study we demonstrate that B cell-restricted anti-CD19 and CD22 SAP immunotoxins are potent and specific reagents for neoplastic B cell elimination. We also show that they can inhibit fresh B-cell chronic lymphocytic leukemia cell proliferation in a short-term DNA synthesis inhibition assay, under conditions that are potentially achievable in vivo. This study addresses several points.

First, CD19 and CD22 antigens are adequate targets for immunotoxin-mediated cytotoxicity. CD19 has already been shown to mediate internalization of the ricin A chain and PAP. Interestingly, conjugates between another anti-CD19 antibody, referred to as B4, and either gelonin or the ricin A chain were ineffective. Both conjugates appear to inhibit normal and neoplastic B-cell protein synthesis. A superior activity of an anti-CD22 compared to an anti-CD19 ricin A chain conjugate has also been reported. We have shown that both CD19 and CD22 immunotoxins actively inhibited DNA and protein synthesis in B cell lines. In a clonogenic assay, in addition, major differences in the killing activity of these immunotoxins on the single cell line were not observed. Based on our data, all three immunotoxins are equally effective in neoplastic B-cell killing.

A spectrum of susceptibility to immunotoxin activity was observed in Burkitt’s lymphoma cell lines. In previous work, we have shown that the clonogenic lymphoma growth after treatment with an anti-IgM SAP immunotoxin was correlated to the expression of the relevant antigen on the cell surface. In the present article we could not find a correlation with CD19 and CD22 antigen expression as they were expressed on most B cell lines. These data, together with the observations of Ghetie on B cells and Bjorn et al on breast cancer cell lines support the notion that the presence of the target epitope on the cell surface cannot by itself predict sensitivity to immunotoxins.

We have used different assays for evaluation of immunotoxin activity: DNA synthesis inhibition, protein synthesis inhibition, and clonogenic limiting dilution assay. A detailed comparison of these methods would be beyond the scope of this study. In our hands, DNA synthesis inhibition assay generally underestimated the activity of the immunotoxins as compared to the other two techniques. Relative advantages of this assay are that it is easy to carry out and can give information on whether the cells are susceptible to the immunotoxin. Protein synthesis inhibition and clonogenic assay, which measures the actual killing of clonogenic tumor cells, may be more reliable methods for immunotoxin evaluation.

Second, we have chosen SAP as the toxic moiety for conjugation in our immunotoxins. SAP shares the same mechanism of action with the ricin A chain as it inactivates 28S eucaryotic ribosomal subunits by modifying both or either of two nucleoside residues. Due to the lack of glycosilated residues, SAP is not rapidly cleared from the circulation by the liver and the reticuloendothelial system.

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**Table 6. ID50 to Anti-B Immunotoxins on Fresh B-CLL cells**

<table>
<thead>
<tr>
<th>UPN</th>
<th>HD37-SAP</th>
<th>HD39-SAP</th>
<th>HD6-SAP</th>
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</thead>
<tbody>
<tr>
<td>82-918</td>
<td>$2.0 \times 10^{-9}$</td>
<td>$9.0 \times 10^{-10}$</td>
<td>$3.0 \times 10^{-10}$</td>
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<tr>
<td>86-745</td>
<td>$2.0 \times 10^{-10}$</td>
<td>$2.3 \times 10^{-10}$</td>
<td>$1.0 \times 10^{-10}$</td>
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</tbody>
</table>

Ficoll-Hypaque-separated cells from two CLL patients (UPN 82-918 and 86-745) were treated with $10^{-12}$ M to $10^{-7}$ M HD37-SAP, HD39-SAP, and HD6-SAP for two hours. ID50 (concentration inhibiting 50% $\text{H-TdR}$ incorporation) was determined for each immunotoxin.
Given its high isoelectric point (pI 10.5), SAP may bear positive charge under physiologic conditions and interact electrostatically with the negatively charged antibody, thus forming a more compact structure in which the linkage is protected from attack by serum enzymes. It also has a more potent activity as compared to ricin A chain. In our previous work, we have shown that analogous anti-immunoglobulin heavy-chain conjugates with ricin A or SAP have both cytotoxic activity against B cell lines. The SAP conjugate, however, could achieve the same cell elimination in less time (two to three hours compared to 12 to 24 hours) and at concentrations inferior to the ricin A chain conjugate. A direct comparison between these anti-CD19 and CD22 conjugates and analogous ricin A-chain immunotoxins has not been made. However, Ghetie et al reported ID50s for HD37-RTA of 1.5 × 10^{-8} to 6.7 × 10^{-11} M for different cell lines after a 24-hour incubation. These figures are somewhat higher than our ID50s of 1 × 10^{-9} to 2.6 × 10^{-12} M for the HD37-SAP after 16-hour incubation. Similar results were obtained with the HD6 conjugates. HD6-RTA gave ID50s of 1.7 × 10^{-9} to 1.4 × 10^{-11} M, while HD6-SAP showed ID50 activity at 4.6 × 10^{-10} to 1.4 × 10^{-12} M. The high protein synthesis inhibitory activity together with the rapid onset of action are important features for the future in vivo use of these immunotoxins.

In this study we report that our immunotoxins actively inhibited proliferation of B-CLL cells, as demonstrated by a new two-step proliferation inhibition assay. Anti-CR1 monoclonal antibody CB04 gave a competence signal to CLL cells which became responsive to low-molecular-weight BCGF. Progression of the cells through the cell cycle to the S-phase was evaluated by DNA synthesis assay. DNA synthesis was measured by the 3H-TdR incorporation method, which gave results superimposable to the originally described bromodeoxyuridine incorporation technique (M. Bregni and S. Venenoni, unpublished results). This assay allowed us to define the activity of SAP immunotoxins on fresh leukemia cells under culture conditions that closely resemble the in vivo situation. SAP immunotoxins were capable of potent and rapid inhibition of B-CLL cell growth. Anti-CD19 immunotoxin was slightly more effective than anti-CD22 immunotoxins. In our series we observed an unexpected high positivity of anti-CD22 antibodies on B-CLL cells, in partial disagreement with data recently reported in the literature. A weak correlation between B-CLL cell-surface antigen expression and percentage of inhibition of DNA synthesis by immunotoxins was also observed. This is not surprising in that DNA synthesis inhibition is a crude estimate of immunotoxins activity. We devised to use this method for its easy applicability. Experiments of comparison between DNA synthesis and protein synthesis inhibition in B-CLL cells are in progress (M. Bregni et al, in preparation).

To our knowledge, few other reports have described the effect of immunotoxins on fresh leukemia cells. Uckun described a CD19-PAP immunotoxin that showed considerable activity on fresh acute lymphoblastic leukemia cells in a soft-agar clonogenic assay. In comparison to that study, we could not define the killing activity of our immunotoxins as we determined inhibition of DNA synthesis. Advantages of our method are that it is easy to perform and does not require careful screening of materials. We also determined immunotoxin activity on fresh hairy-cell leukemia cells, which express CD19 and CD22 surface antigens and can be

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Table 7. Inhibition of DNA Synthesis of B-CLL Cells by Immunotoxin Treatment of Whole Blood

<table>
<thead>
<tr>
<th>UPN</th>
<th>Unstimulated</th>
<th>Stimulated</th>
<th>HD37-SAP</th>
<th>HD39-SAP</th>
<th>HD6-SAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>87-98</td>
<td>42</td>
<td>24,211</td>
<td>2,710 (89)*</td>
<td>5,500 (77)</td>
<td>3,169 (87)</td>
</tr>
<tr>
<td>87-1,188</td>
<td>50</td>
<td>20,589</td>
<td>694 (97)</td>
<td>3,360 (83)</td>
<td>8,333 (60)</td>
</tr>
<tr>
<td>86-1,049</td>
<td>86</td>
<td>47,337</td>
<td>48,333 (0)</td>
<td>42,158 (0)</td>
<td>40,330 (0)</td>
</tr>
<tr>
<td>86-2,214</td>
<td>226</td>
<td>5,532</td>
<td>268 (99)</td>
<td>234 (100)</td>
<td>217 (100)</td>
</tr>
<tr>
<td>80-633</td>
<td>333</td>
<td>1,103</td>
<td>275 (100)</td>
<td>247 (100)</td>
<td>360 (100)</td>
</tr>
<tr>
<td>79-277</td>
<td>494</td>
<td>8,713</td>
<td>244 (100)</td>
<td>282 (100)</td>
<td>269 (100)</td>
</tr>
<tr>
<td>87-656</td>
<td>2,750</td>
<td>13,371</td>
<td>5,032 (79)</td>
<td>6,203 (67)</td>
<td>6,793 (62)</td>
</tr>
</tbody>
</table>

50 nM HD37-SAP, HD39-SAP, and HD6-SAP were added to 1 mL whole CLL blood. After a two-hour incubation, mononuclear cells were separated by Ficoll-Hypaque density gradient and stimulated to incorporate 3H-TdR by incubation with CB04-S and BCGF, as detailed in Table 5.

*\( = \) percent inhibition.

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Fig 5. DNA synthesis inhibition of B-CLL cells by immunotoxin treatment of whole blood or mononuclear cells. B-CLL fresh whole blood of Ficoll-Hypaque separated cells from the same specimen (UPN 87-856) were incubated for two hours with 50 nM HD37-SAP, HD39-SAP, and HD6-SAP. After washings, mononuclear cells from the whole blood samples were obtained by Ficoll-Hypaque separation. 3H-TdR incorporation in treated samples was determined as detailed in Fig 3.
induced to proliferate by stimulation with B-cell growth factor. Anti-B immunotoxins efficiently inhibited hairy-cell leukemia cell growth (M. Bregni et al, in preparation).

B-CLL is a heterogeneous disease with a highly variable clinical course. Many patients do not require specific therapy, while some have a rapidly progressive disease with severe anemia and thrombocytopenia. Most patients develop hypogammaglobulinemia and an impaired response to immunization. Treatment of B-CLL is largely based on single-agent chemotherapy, most commonly the alkylating agent chlorambucil. Many features of the disease (expression of relevant antigens, including the 65kD CD5 molecule, on the cell surface, abnormal immune response, presence of leukemic cells in the blood stream, poor response to second-line chemotherapy) make CLL an attractive candidate for immunotoxin therapy studies. Other investigators have reported on the use of an anti-CD5–ricin A chain immunotoxin, referred to as T101-RTA, for therapy of refractory B-CLL. In their studies, infusion of 3–14 mg/m² of T101-RTA was well tolerated in all cases, with no major toxicities. Clinical response, however, was limited to a rapid and transient fall in WBC count of less than 24 hours duration, most likely due to the antibody portion of the conjugate. Fresh B-CLL cells were resistant to in vitro treatment with T101-RTA at concentrations up to 10⁻⁸ M. A biologic activity of the conjugate could be demonstrated only in the presence of the enhancer human serum albumin-monomers. We show in this study that our B-antigen-targeted SAP immunotoxins can eliminate B-CLL cells in vitro from whole blood with short incubation times, at a low immunotoxin concentration, and in the absence of enhancers. Most important, fresh human plasma components do not affect SAP immunotoxin activity. An anti-CD5-SAP conjugate could also kill B-CLL cells under the same experimental conditions (Siena et al, submitted for publication). Based on our preclinical data, we conclude that our B-cell-restricted SAP conjugates show a good potential for treatment of B-cell leukemia and lymphoma. A phase I and II clinical trial is warranted.

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B-cell restricted saporin immunotoxins: activity against B-cell lines and chronic lymphocytic leukemia cells

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