Systemic Therapy with 3BIT, a Triple Combination Cocktail of Anti-CD19, -CD22, and -CD38-Saporin Immunotoxins, Is Curative of Human B-Cell Lymphoma in Severe Combined Immunodeficient Mice

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ABSTRACT

We demonstrate in these preclinical studies that all severe combined immunodeficient mice injected with the human B-cell lymphoma cell line Ramos are cured when treated with a combination of anti-CD19, -CD22, and -CD38-saporin immunotoxins (ITs; termed 3BIT). Each component IT used individually did not cure the majority of animals but did significantly prolong their survival compared with PBS sham-treated controls, although the majority succumbed eventually to disease. The very significant improvement obtained with the three-IT combination 3BIT was not due to an antibody or antibody-plus-IT effect. We postulate that by targeting against these three cell surface molecules, we have effectively ensured delivery of saporin to each lymphoma cell with growth potential within the tumor, thus overcoming the problems of heterogeneity of target antigen expression that can limit the therapeutic efficacy of single-IT therapy or even two-IT combination therapy. These “proof of principle” findings have an obvious important bearing on antibody-based therapies for cancer and provide the rationale needed for the design and implementation of clinical trials with such combinations.

INTRODUCTION

Heterogeneity of target antigen expression is a major limiting factor that determines the success of any Ab1-based therapy for cancer in which delivery of a cytotoxic agent to all malignant cells with growth potential within the tumor is essential for total tumor ablation. Thus, variant tumor cells that fail to express the target antigen will evade destruction via Ab-mediated delivery of the cytotoxic agent and the subsequent regrowth of these surviving cells will lead to tumor recurrence. This is possibly the most important principle determining the success or failure of these emerging and highly promising types of targeted therapies for malignant disease, and this issue needs to be investigated carefully in preclinical studies if such treatments are to be translated successfully into the clinic. One way of overcoming this problem would be to target against two or more molecules on the tumor cell surface in the expectation that multiple antigen-negative tumor cells would occur at a much lower frequency than single antigen-negative cells. Moreover, there would be the added bonus that targeting against multiple cell surface molecules would deliver greater amounts of the cytotoxic agent to multiple-target antigen-positive tumor cells, thus increasing the likelihood of killing these cells.

Two recent independent studies using ITs have shown that targeting against two different surface molecules (CD19/CD38 and CD19/CD22, respectively) on human B-cell lymphomas growing in SCID mice is therapeutically significantly more effective than targeting against either molecule alone (1, 2). However, in both studies, the two IT cocktails achieved a cure in only a proportion of the animals treated, the remainder succumbing to disease. A 100% cure rate was achieved only when a cocktail of anti-CD19 plus anti-CD22 ricin A-chain ITs was used in combination with any one of the three small-molecule cytotoxic drugs doxorubicin, cytotoxin, or camptothecin in human lymphoma-bearing SCID mice (3). This indicates to us that in these model systems, targeting against only two surface molecules is inadequate for the elimination of all cells with growth potential within the tumor. We postulate that this is likely to be due to the existence of dual antigen-negative cells within the tumor cell population. In this respect, our own study showed that a tiny proportion (0.04%) of the Ramos cell line was negative for both CD19 and CD38 expression, and we speculated that it was from these cells that tumor growth occurred following therapy with anti-CD19 and -CD38 saporin ITs (1). The addition of a third IT to the cocktail that identifies a separate target molecule on the tumor cell surface may overcome this problem, providing that there are no lymphoma cells within the tumor cell population with a triple antigen-negative immunophenotype. We report here that using a cocktail of three saporin ITs (anti-CD19, -CD22, and -CD38) for the therapy of xenografts of Ramos human Burkitt’s lymphoma (4) in SCID mice is 100% effective at eliminating disease from these animals. Because there are no Ramos cells with the immunophenotype CD19−, CD22−, and CD38− within the population, the results of this “proof of principle” preclinical study strongly support our hypothesis as presented and provides a sound rationale for implementing clinical trials with similar combinations in human B-cell malignancies.

MATERIALS AND METHODS

SCID Mice. Pathogen-free CB-17 scid/scid mice of both sexes 6—10 weeks of age were produced from our own breeding colony and used in all of the experimental work described here. All manipulations on experimental animals were carried out in a laminar flow hood, and these animals were maintained in filter-top microisolator cages and provided with sterile food and water ad libitum.

Ramos Cell Line. The cell line Ramos was derived from a patient with Burkitt’s lymphoma and has been described previously (4).

Saporin and Ab Production. Seeds of Saponaria officinalis were supplied kindly by Chiltern Seeds (Ulverston, Cumbria, United Kingdom). The S06 isoform of saporin was extracted and purified to homogeneity from seeds as described previously (5). The Abs BU12 (anti-CD19), 4KB128 (anti-CD22), and OKT10 (anti-CD38) were produced in quantity by growing the appropriate hybridoma clone in an Endotronics (Cellex) Acusyst R hollow-fiber bioreactor system (Cellex, Minneapolis, MN). Ab was purified from hollow-fiber harvests by a combination of ammonium sulfate precipitation followed by anion exchange chromatography on DEAE-Sepharose and gel filtration on Sephacryl S200HR.

IT Construction. The ITs BU12-saporin (1BITa), OKT10-saporin (1BITb), and 4KB128-saporin (1BITc) were constructed by conjugating the appropriate Ab to saporin using the heterobifunctional cross-linking reagent N-succinimidyl-3-(2-pyridyldithio)propionate as described previously (6). Free Ab was removed from the immun conjugates by carboxymethyl-Sepha-
Table 2 ITs and their specificities

<table>
<thead>
<tr>
<th>IT name</th>
<th>Specificity</th>
</tr>
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<tbody>
<tr>
<td>BU12-saporin</td>
<td>Anti-CD19</td>
</tr>
<tr>
<td>OKT10-saporin</td>
<td>Anti-CD38</td>
</tr>
<tr>
<td>4KB128-saporin</td>
<td>Anti-CD22</td>
</tr>
</tbody>
</table>

Table 3 Inhibition of protein synthesis in target Ramos cells by various immunotoxins and saporin

<table>
<thead>
<tr>
<th>ITs</th>
<th>IC$_{50}$a (µg/ml)</th>
<th>Fold increaseb</th>
</tr>
</thead>
<tbody>
<tr>
<td>1BITa</td>
<td>0.057</td>
<td>40</td>
</tr>
<tr>
<td>1BITb</td>
<td>0.13</td>
<td>18</td>
</tr>
<tr>
<td>1BITc</td>
<td>0.011</td>
<td>209</td>
</tr>
<tr>
<td>2BITa</td>
<td>0.012</td>
<td>192</td>
</tr>
<tr>
<td>2BITb</td>
<td>0.049</td>
<td>47</td>
</tr>
<tr>
<td>2BITc</td>
<td>0.011</td>
<td>209</td>
</tr>
<tr>
<td>3BIT</td>
<td>0.014</td>
<td>164</td>
</tr>
<tr>
<td>Saporin only</td>
<td>2.3</td>
<td></td>
</tr>
</tbody>
</table>

*a Calculated as the concentration that inhibits protein synthesis by 50% relative to untreated control cultures.
*b Fold increase relative to saporin alone.

Table 1 Immunophenotypic analysis of CD19, CD22, and CD38 expression by the human Burkitt’s lymphoma cell line Ramos

<table>
<thead>
<tr>
<th>Immunophenotype</th>
<th>Percentage</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD19$^+$, CD22$^+$, and CD38$^+$</td>
<td>99.0</td>
<td>Triple positive</td>
</tr>
<tr>
<td>CD19$^+$, CD22$^+$, and CD38$^-$</td>
<td>99.8</td>
<td>Dual positive</td>
</tr>
<tr>
<td>CD19$^-$, CD22$^+$, and CD38$^+$</td>
<td>99.3</td>
<td>Dual positive</td>
</tr>
<tr>
<td>CD19$^-$, CD22$^-$, and CD38$^+$</td>
<td>99.3</td>
<td>Single positive</td>
</tr>
<tr>
<td>CD19$^+$, CD22$^-$, and CD38$^+$</td>
<td>99.4</td>
<td>Single positive</td>
</tr>
<tr>
<td>CD19$^+$, CD22$^-$, and CD38$^-$</td>
<td>99.9</td>
<td>Single positive</td>
</tr>
<tr>
<td>CD19$^-$, CD22$^-$, and CD38$^-$</td>
<td>0</td>
<td>Triple negative</td>
</tr>
</tbody>
</table>

Protein Synthesis Inhibition Assay. The ability of each IT to inhibit protein synthesis in target Ramos cells was evaluated by a $[^{3}$H]leucine incorporation assay as described previously (8).

SCID Mouse Therapy Studies. The SCID-Ramos model of human B-cell lymphoma has been described by us previously (1). Two million Ramos cells in the logarithmic phase of growth were injected on day 1 via the tail vein into groups of 8 or 10 SCID mice at 6–10 weeks of age. Each therapy group comprised an equal number of male and female animals. Therapy was given on days 7, 9, and 11 and consisted on each occasion of a 200-µl bolus injection of (a) the appropriate single IT (10 µg); (b) a two-IT combination, IT + IT or IT + Ab (5 µg of each); or (c) a three-IT combination, IT + IT + IT, IT + IT + AB, or IT + AB + AB (3.3 µg of each). Details of the precise composition of each therapy group are given in Table 4.

All animals were monitored daily for the 300-day duration of the study, and animals showing adverse effects or appearing unwell due to tumor growth were killed, and full postmortem examinations were conducted. The presence of tumor was confirmed at postmortem examination and the activity of each IT was evaluated on the basis of tumor burden at 300 days after treatment. The results were analyzed using the Mann-Whitney test (9).
of Ramos tumors was confirmed, in sacrificed animals and those animals dying intercurrently, by conventional histopathology and/or flow cytometry. Tumors were taken from the majority of animals dying with tumor, and single-cell suspensions were prepared and subjected to flow cytometric analysis for quantification of CD19, CD22, and CD38 expression. Animals surviving to 300 days were killed at this time; full postmortem examinations were carried out; and portions of spleen, liver, lungs, brain, and kidneys were taken for each individual Ab and visualized with FITC-labeled rabbit antimouse immuno- 

cytokine staining.

CD22, and CD38 cell surface expression by single-color flow cytom

tery as shown in Fig. 1A. Expression levels could be ranked as

 attrition rate was intermediate between 1BITa and 1BITc. Comparison

result in any long-term survivors. Only 10% of animals treated with

therapy with each of the individual ITs is shown in Fig. 2A, and the

combination cocktails. This was particularly true for 3BIT, which achieved an IC50

ranked in the following order of potency: 1BITc (CD22) > 1BITa (CD19) > 1BITb (CD38). Although 1BITa had an IC50 lower than

of 0.014 pg/ml (representing a 164-fold increase in cytotoxicity

calculated IC50s reported in Table 3, the individual ITs could be

determined in the SCID-Ramos model described by us previously (1).

In Vitro Effects of Single IT and Two- and Three-IT Combinations on Protein Synthesis in Ramos Cells. The three individual saporin ITs with anti-CD19, -CD22, and -CD38 specificities are detailed in Table 2. To determine the in vitro effects of each of these individual ITs or combinations of two or all three ITs on protein synthesis in the Ramos cell line, we exposed cultures of these cells to increasing concentrations of each as shown in Fig. 1B. On the basis of calculated IC50s reported in Table 3, the individual ITs could be ranked in the following order of potency: 1BITc (CD22) > 1BITa (CD19) > 1BITb (CD38). Although 1BITa had an IC50 lower than

molecules, and there were no cells negative for all three target mol

dies, and there were no cells negative for all three target molecules.

Survival of SCID-Ramos Mice following Single-IT or Two- and Three-IT Combination Therapy. The therapeutic efficacies of each individual IT or the various combinations of two or three were determined in the SCID-Ramos model described by us previously (1). The i.v. injection of 2,000,000 Ramos cells into nonconditioned SCID mice is invariably fatal and leads to the death of animals over a highly predictable time course. The survival of SCID-Ramos mice receiving therapy with each of the individual ITs is shown in Fig. 2A, and the survival statistics are summarized in Table 4. The ITs could be ranked in order of therapeutic effectiveness as 1BITc > 1BITb > 1BITa, with 60% long-term disease-free survivors obtained with 1BITc. In contrast, treatment with 1BITa, although prolonging survival, did not result in any long-term survivors. Only 10% of animals treated with 1BITb survived for the 300-day duration of the study, and the disease attrition rate was intermediate between 1BITa and 1BITc. Comparison
by log-rank analysis of sham-treated control animals with each of the three individual IT treatment groups revealed that all three treatments exerted a statistically significant therapeutic effect.

Survival of SCID-Ramos mice treated with the three possible combinations of two ITs (2BITa, 2BITb, and 2BITc) or with a combination of all three (3BIT) is shown in Fig. 2B and summarized in Table 4. The therapeutic efficacy of these various IT combinations could be ranked as follows: 3BIT > 2BITa > 2BITb = 2BITc. All animals treated with the triple-IT combination 3BIT survived disease free for the 300-day duration of the study. Of the three two-IT combination treatments, 2BITa performed best, with only two animals developing tumor at 189 and 202 days, respectively. 2BITb and 2BITc performed almost identically, with a similar disease attrition rate and with half of the animals in both groups surviving disease-free for the 300-day duration of the study. Log-rank analysis demonstrated that all therapeutic outcomes were highly significantly different from sham-treated controls and moreover demonstrated significant differences between the various combination therapy groups, which are summarized in Table 5. All surviving animals were disease free, with no tumors detectable grossly, by immunocytochemistry or by flow cytometry on termination of the study at 300 days.

Single and Combination Ab Therapy. The effects of therapy with native Ab used singly (1BABa, 1BABb, and 1BABc) or in combinations of two (2BABa, 2BABb, and 2BABc) or three (3BAB; therapy groups 8—14) had only very minor effects on survival length, with no long-term survivors in any of the treatment groups (Table 4). None of these results were significantly different by log-rank analysis from PBS sham-treated controls.

Combinations of IT and Ab. Experiments were undertaken to exclude the possibility of an interaction between an Ab component(s) and IT component(s) as being responsible for the significant improvements to therapy outcome. The survival statistics for groups of SCID-Ramos mice treated with the possible combinations of a single IT plus single Ab (groups 15—19), a single IT plus two Abs (groups 20—22), or two ITs plus a single Ab (groups 23—25) are summarized in Table 4. Importantly, all of these control groups performed significantly worse than 3BIT.

Analysis of in Vivo Tumor Immunophenotype following IT Therapy. Tumors emerging in IT-treated animals were analyzed for the levels of CD19, CD22, and CD38 expression by single-color flow cytometry and data representative of the overall results obtained are shown in Fig. 3. Analysis of tumors occurring in PBS sham-treated control animals also revealed a similar reduction in levels of expression of all three antigens.
COMBINATION IMMUNOTOXIN THERAPY OF HUMAN LYMPHOMA

A COMBINATION IMMUNOTOXIN THERAPY OF HUMAN LYMPHOMA

A

JAW / NASAL TUMORS

% WITH TUMOR

0 20 40 60 80 100

ITs → Aba → Ab + IT combination → PBS

TREATMENT GROUP

B

BRAIN / CNS TUMORS

% WITH TUMOR

0 20 40 60 80 100

ITs → Aba → Ab + IT combination → PBS

TREATMENT GROUP

C

ABDOMINAL TUMORS

% WITH TUMOR

0 20 40 60 80 100

ITs → Aba → Ab + IT combination → PBS

TREATMENT GROUP

D

KIDNEY TUMORS

% WITH TUMOR

0 20 40 60 80 100

ITs → Aba → Ab + IT combination → PBS

TREATMENT GROUP

Fig. 4. Percentage of animals within each therapy group (groups 1-25) and in PBS sham-treated controls (group 26) developing tumors in the jaw/nasal region (A), brain/CNS (B), abdomen (C), or kidney (D). Percentages are calculated on the basis of the total number of animals within each group that succumb eventually to a tumor at any site.

sham-treated animals but were a very common feature in IT or IT-plus-Ab combinations (Fig. 4A). Treatment with single or combination Abs also led to a smaller number of jaw/nasal tumors. The converse was true for CNS/brain tumors that occurred frequently in PBS sham-treated and Ab treatment groups but far less frequently in IT or IT-plus-Ab groups (Fig. 4B). There was no clear influence of therapy on the distribution patterns of tumors occurring at other sites (Fig. 4, C and D).

DISCUSSION

The results presented here show that a therapeutically superior result is obtained with the triple-IT combination 3BIT, with 100% disease-free survivors at 300 days. The outstanding therapeutic performance of 3BIT was not due to an Ab or Ab(s)-plus-IT(s)-mediated effect, inasmuch as appropriate control groups did not show the same therapeutic outcome. This is quite different from the findings of Ghetie et al. (2), who showed that an anti-CD22 ricin A-chain IT used in combination with an anti-CD19 Ab was as effective in a SCID mouse model of human lymphoma as both equivalent ITs used in combination. Here, it was shown that anti-CD19 Ab alone exerted a direct growth-inhibitory signal that, together with the direct cytotoxic effects of the anti-CD22 IT, led to an improved therapeutic performance. Such an effect was not demonstrable in the current study nor in a previously study described by us (1). It is also of interest that the in vitro protein synthesis assay did not predict the in vivo therapeutic improvement obtained with 3BIT. This probably reflects the fact that this short-term assay is incapable of detecting and accounting for the minute single antigen-negative tumor cell populations at the 48-h time point. This being the case, the tiny triple antigen-positive Ramos subpopulation would make little discernible contribution to observed protein synthesis levels when 3BIT was used within this time frame. Only a longer-term clonogenic or outgrowth-type assay would be capable of detecting this type of effect (1).

With IT, it is essential to deliver toxin to the interior of each target cell that has growth potential within the tumor. Tumor cells that are negative or down-regulated for a single target molecule would not be killed by an IT targeting against this single antigen. By targeting against more than one cell surface antigen, the probability of delivering a lethal dose of toxin is increased (a) because of the higher probability that all tumor cells within the population would be positive for at least one of the target molecules and (b) because greater quantities of toxin would be delivered to those tumor cells that expressed more than one of the target molecules. It follows from this that the more target molecules aimed against on the tumor cell surface, the greater would be the probability of killing every individual cell in the tumor. Indeed, it is entirely feasible that cocktails of ITs could be custom blended for an individual patient's tumor, with the specificities in the cocktail determined by the immunophenotype of the tumor cell population.

Tumors occurring in single- or two-IT treatment groups were found to still express the target molecule(s) against which therapy was directed, although some modest down-regulation of expression was encountered in the majority of cases. However, such down-regulation was also encountered in PBS sham-treated animals, and we conclude
that this is therefore likely to be associated with a direct influence of the SCID mouse microenvironment on antigen expression, a phenomenon also described by others (9). Why is it, therefore, that tumor cells expressing the relevant target molecules were able to escape killing by IT? There are at least three possibilities: (a) a fraction of the tumor cells may have been located within the animal at a site inaccessible to IT; (b) a small fraction of tumor cells may have been down-regulated for antigen expression at the time of IT treatment and thereby avoided killing by IT and upon emergence these escapee cells up-regulated antigen expression; and (c) target antigen-positive tumors may represent selection of an IT-resistant subclone. The first possibility can be excluded, given that highly effective treatment with 3BIT demonstrates clearly that all tumor cells within the mouse with growth potential must be accessible to IT. The third possibility is also unlikely, because we have shown in previous studies that Ramos tumors emerging in animals treated with ITs are still sensitive in vitro to IT.4 We therefore feel that the second possibility of down-regulation of target antigen by a small subpopulation of tumor cells at the time of treatment offers the most likely explanation for this observation.

The suppression of CNS disease by IT, but not Ab therapy, is surprising in view of the blood-brain barrier that should prevent access of a large molecule such as an IT into the CNS. An explanation for this could lie with the possibility that CNS disease might not arise as a direct result of lymphoma cell seeding of the CNS but from infiltration into the CNS by lymphoma cells directly from the bone marrow cavity. In these circumstances, the modifying effects of IT could be viewed as elimination of bone marrow-based disease by IT, leaving the tumor to grow at other sites (i.e., jaw and abdomen), where total elimination was unsuccessful.

The preclinical studies described here have demonstrated proof of principle using an in vivo model of human lymphoma, albeit that the Ramos cell line possesses a relatively homogeneous immunophenotype. When considering individual primary lymphomas and leukemias in patients, the heterogeneity of antigen expression will be considerably greater than that encountered for any established cell line. It follows, therefore, that application of this principle to patients is likely to be more problematic. Nonetheless, an important principle has been demonstrated and is in a broader sense applicable to other malignancies beside lymphoma. It is feasible to envisage a customized IT cocktail for individual patients, the individual component Abs of which would be based on the immunophenotype of the global tumor cell population. There are, of course, other important factors that are likely to limit the efficacy of such therapeutic interventions. Among these we can include tumor penetration by the IT(s), suitability of the target molecule(s), pharmacokinetics of individual ITs in the cocktail, internalization and trafficking issues, immunogenicity of the conjugates, and, importantly, dose-limiting toxicities that may prevent the achievement of effective therapeutic levels of IT in the patient. Improving the IT therapeutic index is an important objective that would help deal with some of these issues, and it seems likely that the utility of IT combinations is likely to make a contribution in this area.

ACKNOWLEDGMENTS

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Systemic Therapy with 3BIT, a Triple Combination Cocktail of Anti-CD19, -CD22, and -CD38-Saporin Immunotoxins, Is Curative of Human B-Cell Lymphoma in Severe Combined Immunodeficient Mice


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