RAPID COMMUNICATION

Eradication of Minimal Disease in Severe Combined Immunodeficient Mice With Disseminated Daudi Lymphoma Using Chemotherapy and an Immunotoxin Cocktail

By M.-A. Ghetie, K. Tucker, J. Richardson, J.W. Uhr, and E.S. Vitetta

Severe combined immunodeficient (SCID) mice injected intravenously with a human Burkitt's lymphoma cell line (Daudi) develop disseminated lymphoma (SCID/Daudi), which is fatal in 100% of the mice. Early treatment of these mice with either an immunotoxin (IT) cocktail (consisting of anti-CD19–ricin A chain plus anti-CD22–ricin A chain) or chemotherapy significantly prolonged survival but was not curative. Combination therapy with the IT cocktail and any one of three chemotherapeutic drugs (doxorubicin, cytoxan, or camptothecin) cured the mice. Cure was demonstrated by both histopathologic examination of treated mice and, more importantly, by adoptive transfer of cells from organs of the cured mice to naive SCID mice where 100 tumor cells would have caused disease in the recipients. These results provide a strong rationale for combining IT therapy with conventional chemotherapy in the treatment of B-cell neoplasia.

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CONVENTIONAL APPROACHES to the treatment of B-cell lymphoma have emphasized the use of cytotoxic agents that can cure 30% to 50% of high-grade lymphomas, but not low-grade lymphomas.1,2 We have used our severe combined immunodeficient (SCID/Daudi) model of disseminated human Burkitt's B lymphoma3 for preclinical evaluation of different immunotoxin (IT) constructs containing either anti-CD22 or anti-CD19 antibodies and deglycosylated ricin A chain (dgA).4,5 These ITs have also been used in over 100 patients in phase I/II clinical trials in patients with refractory non-Hodgkin's lymphoma (NHL)6,7 and (Stone et al, unpublished data; Sausville et al, unpublished data, Kaplan et al, unpublished data, 1994).

Our previous studies have shown that neither Fab' nor IgG anti-CD22 (RFB4)-dgA4 or IgG anti-CD19 (HD37)-dgA cured SCID/Daudi mice with early disease, although survival was significantly prolonged. A combination of the two ITs or a combination of anti-CD22-dgA and anti-CD19 antibody (without dgA) extended survival of SCID/Daudi mice significantly longer than either treatment alone. Thus far, the only combination treatment that has been curative in SCID mice was a large amount of anti-CD19 antibody and the anti-CD22–immunotoxin, RFB4-dgA. The mice treated with this combination survived more than 1 year, at which time they appeared tumor free.8 In the present study we compare the therapeutic effect of combination treatment with chemotherapy and ITs. We selected three chemotherapeutic drugs that are commonly used in humans: cytoxan (cyclomiphosphamide), doxorubicin, and methotrexate, as well as a less commonly used drug, camptothecin, which has recently been re-evaluated for the therapy of human carcinomas.9-12

Neither doxorubicin nor methotrexate has been used in combination with ITs. The most significant therapeutic effect of either drug alone was achieved using an immunoon conjugate containing doxorubicin and the BR96 antibody. This conjugate cured xenografted human lung, breast, and colon carcinomas grown subcutaneously in athymic mice. This conjugate also cured 70% of mice with metastatic human lung carcinoma.13 Liposome-encapsulated doxorubicin has also been used to eradicate lung cancer in mice.14,15 The toxicity of doxorubicin to the gastrointestinal tract and myocardium is reduced when the drug is administered in encapsulated form. In a phase I clinical trial, TLCD-99 (doxorubicin encapsulated in liposomes) was well-tolerated and produced fewer side effects than would be expected with nonencapsulated drug at equal doses.16

Clinical trials with camptothecin in its water-soluble form were discontinued because of severe toxicity.9,12 However, new derivatives of camptothecin have been used with some success in phase II clinical studies in patients with refractory leukemia and lymphoma17 and in phase I and II studies in patients with advanced non-small cell lung carcinoma18 or with gynecologic cancers.19 Complete inhibition of the growth of human malignant melanoma cells in vitro and regression of human melanoma xenografts, breast xenografts,11 and ovarian carcinomas20 in nude mice have also been induced with camptothecin. These studies suggested that a combination of ITs and different chemotherapeutic regimens might improve the efficacy of ITs reactive with human B lymphoma.

This study shows that therapy with a cocktail of two ITs (anti-CD22-dgA and anti-CD19-dgA) plus a single chemotherapeutic agent cures human Burkitt's lymphoma (Daudi cells) in SCID mice with no evidence of minimal residual disease or dormant tumor cells.

MATERIALS AND METHODS

Cells. The Burkitt's lymphoma cell line Daudi was maintained in culture by serial passage in RPMI 1640 medium containing 25 mM/L HEPES, 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 100 mM/L L-glutamine (complete medium). The cells were grown in a humidified
atmosphere of 5% CO₂ and air. Cells were used for intravenous (IV) inoculation of SCID mice. Cell viability was determined by trypan blue exclusion.

Animals. Female SCID mice (CB-17 SCID/SCID) were purchased from the Harlan Sprague Dawley Laboratory (Madison, WI). They were housed and maintained in a specific pathogen-free (SPF) facility. Animals were fed autoclaved food and sterile water ad libitum, and all manipulations were performed in a laminar flow hood.

Induction of human tumor in SCID mice. Six- to 10-week-old SCIDs were inoculated IV with 5 × 10⁶ Daudi cells in 0.1 mL phosphate-buffered saline (PBS). Mice were monitored daily and killed at the onset of paralysis of both hind legs, a clinical symptom that precedes death.3 (The mean paralysis time [MPT] has been established as the end point in this animal model.) Complete necropsy and histopathologic examination were performed on several animals in each experimental group. Tissue from lung, heart, liver, spleen, kidney, ovary, and vertebrae were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Therapy of SCID/Daudi mice. SCID mice were inoculated with 5 × 10⁶ Daudi cells 24 hours before treatment. In each experiment, four groups of 5 to 10 mice with an average weight of 20 g were used and treatment was administered on days 1 through 4 after tumor inoculation as follows: (1) control mice were injected retroorbitally (RO) with PBS or saline; (2) mice were injected RO with an IT cocktail (RFB4-dgA [60 μg] + HD37-dgA [60 μg]); (3) mice received either one of the following chemotherapeutic drugs: methotrexate (112 μg), doxorubicin (80 μg), cytoxan (1.6 mg), or camptothecin (640 μg) (all chemotherapeutics but camptothecin were administered IV on days 1 through 4 divided into four equal amounts; camptothecin was injected intramuscularly in IM) in an emulsion in intralipid two times per week for 4 weeks; (4) mice were administered a combination of treatments (2) and (3) as described above. When therapy was initiated, the SCID mice had 1 × 10⁷ disseminated Daudi cells as described elsewhere.3

Adaptive transfer. Mice surviving for 150 to 200 days were killed and cells from ovaries and spinal cords were injected IV into healthy SCID mice. Adaptive recipients were followed-up for 200 days. At this time, the transfer of 100 tumor cells should have caused death in the recipients.3

Cytotoxic effect of different chemotherapeutic drugs alone or in combination with IT. A Daudi killing assay for each drug (methotrexate, doxorubicin, cytoxan, and camptothecin) was performed as described elsewhere.29 The inhibition of ³H-thymidine incorporation was used to determine individual IC₅₀ (M). The cytotoxic effect of a cocktail containing different amounts of anti-CD22–dgA and a constant amount of anti-CD19–dgA (eg, the IC₅₀) dose and vice versa was also determined and compared with combination treatment using each IT plus either one of the chemotherapeutic drugs at a certain concentration (eg, the IC₅₀ dose). To determine whether the cytotoxic effect of combination treatment was additive or synergistic, an analysis using an algebraic method was performed and the combination index (CI) was calculated using the equation: CI = A/Ae + B/Be < 1 (synergistic); = 1 (additive), where A and B are the concentrations of the two drugs that, in combination, kill 90% of the Daudi cells, and Ae and Be are the concentrations of the two drugs extrapolated from their individual killing curves and corresponding to 90% killing.31

Immunofluorescence assay. The phenotype of Daudi tumor grown in SCID mice has been previously described.3 To determine whether treatment with drugs generated CD22- or CD19- Daudi cells, we harvested tumors from doxorubicin-treated mice (ovary and spine) and as well as from untreated SCID/Daudi mice (ovary). Single-cell suspensions were prepared and cells were analyzed by indirect immunofluorescence, using the parental Daudi cells as a control.30 Cells were stained with the primary antibodies anti-CD22 and anti-CD19 followed by a fluorescein isothiocyanate (FITC)-labeled goat-antimouse Ig (GAM Ig). The cells were analyzed on a fluorescence-activated cell sorter (Becton-Dickinson, Oxnard, CA).

RESULTS

Cytotoxic effect of different chemotherapeutic agents on Daudi cells. The chemotherapeutic drugs used in the SCID/Daudi model are described in Table 1. These drugs (methotrexate, cytoxan, doxorubicin, and camptothecin) induce antitumor effects by interacting with cellular DNA. As shown in the table, methotrexate did not kill Daudi cells whereas doxorubicin and camptothecin had comparable IC₅₀ (10⁻⁹ mol/L) and cytoxan was less potent (IC₅₀ = 10⁻⁴ mol/L).

Antitumor effect of different drugs in SCID/Daudi mice. Two different doses (20% and 40% of the LD₅₀ dose) were administered to separate groups of SCID/Daudi mice. As shown in Table 2, methotrexate did not prolong survival of SCID/Daudi mice at the 40% LD₅₀ dose whereas the other three drugs gave dose-dependent prolongation of survival. Therefore, 40% of the LD₅₀ dose of cytoxan or doxorubicin or 640 μg camptothecin was used in subsequent experiments, because this dose induced a significant prolongation of MPT in SCID/Daudi mice. Furthermore, at this dose mice did not show toxic effects as determined by histopathologic examination.

Phenotype of Daudi tumor in SCID mice treated with

| Table 2. Antitumor Effect of Different Chemotherapeutic Drugs in SCID/Daudi Mice |
|------------------|------------------|------------------|
| Drug             | Treatment (dose)* | MPT ± SD (d)     |
| Control          | No treatment     | 31.7 ± 2.6 (10)  |
| Methotrexate     | 40% LD₅₀ (112 μg) | 31.6 ± 2.4 (5)   |
| Cytoxan          | 20% LD₅₀ (0.8 mg)| 51.8 ± 4.1 (5)   |
|                   | 40% LD₅₀ (1.6 mg)| 63.4 ± 12.7 (11) |
| Doxorubicin      | 20% LD₅₀ (40 μg)| 31.2 ± 1.2 (11)  |
|                   | 40% LD₅₀ (80 μg)| 62.2 ± 13.8 (27) |
| Camptothecin     | 40 μg × 8 = 320 μg | 44.4 ± 9.7 (7)   |
|                   | 80 μg × 8 = 640 μg | 74.7 ± 17.6 (12) |

* All drugs but camptothecin were injected IV in four equal doses on days 1-4 after injection with tumor cells; camptothecin (sonicated in intralipid) was administered IM 2 times per week for 4 weeks.
† The number in parentheses is the number of mice in each group.

Table 1. Cytotoxic Effect of Different Chemotherapeutics on Daudi Cells In Vitro and on SCID Mice

<table>
<thead>
<tr>
<th>Chemotherapeutic</th>
<th>Category</th>
<th>IC₅₀ (mol/L)</th>
<th>LD₅₀ Dose (mg/kg)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methotrexate</td>
<td>Antimetabolite</td>
<td>Not toxic up to 2.2</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>× 10⁻³ mol/L</td>
<td></td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>Antibiotic</td>
<td>4.2 ± 13 × 10⁻⁴ (3)†</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.8 ± 16 × 10⁻⁴ (3)†</td>
<td>200</td>
</tr>
<tr>
<td>Cytoxan</td>
<td>Alkylating agent</td>
<td>1.1 ± 0.2 × 10⁻⁴ (3)†</td>
<td>ND</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.
* Two mice were used to determine the LD₅₀.
† The number in parentheses is the number of experiments performed.

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doxorubicin. Daudi cells from SCID mice treated with 40% of the LD$_{50}$ dose of doxorubicin were analyzed for their CD19/CD22 phenotype. As shown in Table 3, residual tumor cells from treated mice had the same phenotype as parental Daudi cells and tumor cells from untreated mice. Hence, chemotherapy did not change the expression of the target antigens for the two ITS.

Effect of combination therapy of SCID/Daudi mice. Chemotherapeutic drugs (doxorubicin, cytoxan, or camptothecin) significantly extended the MPT of treated mice as did treatment with a cocktail of the two ITS (anti-CD22-dgA + anti-CD19-dgA). However, at the time of killing these mice showed the same pattern of tumor growth as control mice. The mice treated with any one of the chemotherapeutic drugs in combination with the cocktail of the two ITS survived over 150 days with no sign of paralysis (Fig 1). When gross and histopathologic examination was performed, none of these mice showed any sign of tumor. At least 10$^7$ cells from ovary and spine were adoptively transferred into naive SCID mice. There was no evidence of tumor growth in recipients after 7 months, at which time the experiments were terminated. Because 100 Daudi cells are sufficient to cause progressive tumor in all the adoptive recipients at 5 months, we considered the original donor mice to be cured.

Synergistic effect of treatment with a cocktail of ITS plus chemotherapeutic drugs in Daudi cells. Both ITS have a significant cytotoxic effect on Daudi cells with IC$_{50}$s of 5.0 $\times$ 10$^{-12}$ mol/L (RFB4-dgA) and 4.5 $\times$ 10$^{-11}$ mol/L (HD37-dgA). The combination of the two ITS (Fig 2A) or each of the two ITS together with a single chemotherapeutic drug had an enhanced cytotoxic effect, which is shown in Fig 2, B and C. Figure 2D shows the cytotoxic effect of each chemotherapeutic drug. The analysis (algebraic method) of the cytotoxic effect of combination treatment shows that the addition of chemotherapeutic drugs to the two ITS induced a synergistic cytotoxic effect on Daudi cells because the CI was <1 for 90% killing. The data in Table 4 show that the combination of the two ITS or one IT plus one chemotherapeutic drug increases the cytotoxic effect on Daudi cells and that the cytotoxic effect is synergistic.

DISCUSSION

The objective of the present study was to compare the antitumor activity of different chemotherapeutic drugs alone or in combination with the two ITS used in other studies in SCID/Daudi mice as well as in clinical trials (and Stone et al. 1989).
Fig 2. Cytotoxic effect of combination treatment was analyzed on Daudi cells (1 x 10^5/well/200 μL) as follows: (A) anti-CD22-dgA (○); anti-CD19-dgA (■); anti-CD22-dgA (2.8 x 10^{-4} to 2.8 x 10^{-6} mol/L) plus anti-CD19-dgA at 2.8 x 10^{-11} mol/L (△); anti-CD19-dgA (2.8 x 10^{-8} to 2.8 x 10^{-10} mol/L) plus anti-CD22-dgA at 2.8 x 10^{-11} mol/L (●); (B) anti-CD22-dgA alone (⊙) or anti-CD22-dgA (2.8 x 10^{-10} to 2.8 x 10^{-12} mol/L) plus doxorubicin at 9.2 x 10^{-8} mol/L (▲); or plus cytoxan at 9.0 x 10^{-8} mol/L (□); or plus camptothecin at 1.4 x 10^{-10} mol/L (◇); (C) anti-CD19-dgA alone (◇) or anti-CD19-dgA (2.8 x 10^{-8} to 2.8 x 10^{-10} mol/L) plus doxorubicin at 9.2 x 10^{-8} mol/L (▲); or plus cytoxan at 9.0 x 10^{-8} mol/L (□); or plus camptothecin at 1.4 x 10^{-10} mol/L (◇); (D) doxorubicin (○); camptothecin (◇); cytoxan (▲). The plates with cells were incubated for 24 hours, pulsed with [3H]-thymidine for 18 hours, harvested, and counted. The reduction of incorporation of cells treated with ITs (percent of control) was plotted against the concentration.

et al, unpublished data; Sausville et al, unpublished data, Kaplan et al, unpublished data, 1994). In a previous study, we showed that SCID/Daudi mice treated with a combination of anti-CD22-dgA (20% of LD50 dose) and large amounts of F(ab′)2 fragments of anti-CD19 antibodies were tumor-free 1 year after treatment. The present study was undertaken in an attempt to find less expensive but equally effective curative therapies for minimal disease in SCID/Daudi mice. Our major finding is that chemotherapy and immunotoxins have synergistic antitumor activity in vitro and cure SCID mice with disseminated B lymphoma.

The in vitro studies of Daudi cells treated with methotrexate, doxorubicin, cytoxan, or camptothecin showed that: (1) methotrexate was not effective at killing Daudi cells either because it must be metabolized by the liver to be active or the Daudi cells are naturally resistant to this drug; (2) Daudi cells were very sensitive to both doxorubicin and camptothecin, with an IC50 of 10^{-7} mol/L; (3) cytoxan was less cytotoxic for Daudi cells and its lower toxic effect (IC50 = 10^{-3} mol/L) was consistent with its higher LD50 in mice.

The doses chosen for in vivo studies (20% and 40% of the LD50 doses) for methotrexate, doxorubicin, and cytoxan, and 640 μg for camptothecin were administered safely with no signs of toxicity. Because methotrexate did not extend the MPT at 40% of the LD50, it was not used in combination with the ITs in vivo.

In a previous study we have shown that a cocktail of two ITs (anti-CD22-dgA and anti-CD19-dgA) extended survival of SCID/Daudi mice in a manner consistent with the killing of 6 logs of tumor cells (50% of mice survived over 100 days). In this study, we combined the two therapies known to be effective individually and show that the combination of the two ITs and one of the above chemotherapeutic agents had a synergistic effect in vitro and eradicated disseminated minimal disease in vivo. Our studies confirm and extend earlier reports that ITs and mafosfamid eliminated...
neoplastic T cells from autologous marrow grafts before bone marrow transplantation and induced a greater antitumor effect than either therapy alone in human solid tumors (T-ALL) grown in nude mice.

In addition, an anti-CD19– pokeweed antiviral protein (PAP) IT in combination with ciclophosphamide was highly effective against human t(4;11) leukemia and CALLA+ human pre-B ALL in SCID mice.

Doxorubicin has not been used previously in combination therapy with ITs, but it has been shown that immunocjugates containing doxorubicin and a tumor-reactive antibody cured several xenografted human tumors in athymic mice. The toxicity of doxorubicin in SCID mice and humans has been circumvented by multiple injections at lower doses. The fact that mice treated with combination therapy (cocktail of ITs and doxorubicin) were tumor free at 200 days was supported by the adoptive transfer experiment which gave no evidence for the presence of residual tumor cells.

In summary, our results support the rationale of using chemotherapy together with an immunotoxin cocktail to treat early and/or minimal disease.

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REFERENCES


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