Antitumor Activity of Anti-CD30 Immunotoxin (Ber-H2/Saporin) In Vitro and in Severe Combined Immunodeficiency Disease Mice Xenografted With Human CD30+ Anaplastic Large-Cell Lymphoma

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To develop a novel adjunctive therapy for CD30 (Ki-1)+ anaplastic large-cell lymphoma (ALCL), we investigated in preclinical studies the antitumor activity of an immunotoxin (IT) constructed by coupling the plant ribosome-inactivating protein saporin to the monoclonal antibody (MoAb) Ber-H2 that is directed against the CD30 molecule, a new member of the tumor necrosis factor receptor (TNFR) superfamily. The activity of Ber-H2/SO6 IT was tested both in vitro against the CD30+ ALCL-derived cell line JB6 and in vivo using our severe combined immunodeficiency disease (SCID) mouse model of human xenografted CD30+ ALCL. In vitro, the Ber-H2/SO6 IT was selectively and highly toxic to the JB6 cell line [50% inhibiting concentration (IC50) = 3.23 × 10−12 mol/L as SO6]. In vivo, a 3-day treatment with nontoxic doses of Ber-H2/SO6 (50% of LD50) induced lasting complete remissions (CR) in 80% of mice when started 24 hours after tumor transplantation. In contrast, injection of the IT at later stages of tumor growth (mice bearing subcutaneous tumors of 40- to 60-mm3 volume), induced CR in only 6 of 21 (approximately 30%) mice and significantly delayed tumor growth rate (P < .01). This finding suggests that maximum effect of the anti-CD30 IT is observed when tumor cell burden is small. Persistent tumors from IT-treated mice consisted of CD30+ cells, thus excluding the possibility that selection of CD30-negative mutant clones during IT therapy was responsible for resistance to treatment. We conclude that Ber-H2/SO6 IT is an effective agent against CD30+ ALCL growing in SCID mice, suggesting its possible role as adjuvant therapy in patients with CD30+ ALCL refractory to standard treatments.

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MATERIALS AND METHODS

Cells

The JB6 cell line was established by in vitro culture of peripheral blood leukemic cells from a 12-year-old boy with advanced, treatment-resistant ALCL expressing a T-cell phenotype and the distinc-

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0006-4971/95/$3.00/0
tive chromosome translocation t(2;5) (p23;q35). Cells were maintained by serial passage in complete RPMI 1640 medium (GIBCO-Biosciences, Paisley, UK) supplemented with 15% (vol/vol) heat-inactivated fetal bovine serum, penicillin (100 IU/mL), streptomycin (100 μg/mL), fungozone (0.25 μg/mL), and L-glutamine (2 mmol/L). Cell cultures were incubated at 37°C in a humidified 5% CO₂ atmosphere.

The HD-derived L428 and L540 cell lines were grown in a similar manner. The L540 cell line required 50 U/mL of interleukin-2 (IL-2; Glaxo, Geneva, Switzerland) for optimal growth conditions.

SCID/JB6 Mice

Development of human CD30⁺ ALCL in SCID mice was originally obtained by intraperitoneal (IP) injection of 10⁶ JB6 cells into four animals; this resulted in a widespread tumor in one mouse. For the propagation of solid tumors, an abdominal mass was removed and minced into Hanks' balanced salt solution (HBSS) medium. Five to six small tissue fragments (2 to 3 mm in diameter) were implanted subcutaneously (SC) into the right flank of carrier mice with a trochar. The fragments produced palpable tumors in more than 90% of mice by day 7, and no variation in engraftment or in kinetics of growth was noted throughout the study. Even the largest tumors did not have a visible adverse effect on the host mice. Tumor volume was determined from the equation: volume = d × D × π/2, where d and D designate shorter and longer tumor diameter, respectively.

MoAbs

Mouse MoAbs specific for CD30 (Ber-H2) and for the B-cell antigen CD22 (OM124) were used in this study. Both antibodies (mouse IgG1 subclass) were purified from hybridoma culture supernatant by affinity-chromatography on protein A-Sepharose CL-4B, as previously described. The antibody preparation of Ber-H2/S06 IT was assessed on both cytospin preparations from the JB6 cell line LCL was used as an irrelevant target for Ber-H2/S06. The in vitro toxicity of Ber-H2/S06 IT was also tested in parallel conditions by linking S06 to the purified Ber-H2 or OM124 with 2-iminothiolane and were separated from the unreduced components by gel filtration on a Sephacryl S-200 high resolution column, as previously described.

Reactivity of Ber-H2/S06 IT

Frozen sections from a variety of normal human tissues of lymphomatoepoietic, epithelial, mesenchymal, or neuroectodermal origin were tested for their reactivity with Ber-H2/S06 IT. The ability of Ber-H2/S06 IT to bind CD30⁺ JB6 cells was assessed on both cytospin preparations from the JB6 cell line and on frozen sections from CD30⁺ JB6 tumor samples in SCID mice. The immunoalkaline phosphatase (AAPAAP) technique was used as detection system. Briefly, cytospins and frozen sections were incubated with the Ber-H2/S06 IT or the native Ber-H2 MoAb (positive control), followed by rabbit anti-mouse Ig (Dako, Glostrup, Denmark) and APAAP immune complexes. All steps were for 30 minutes with washes in 0.05 mol/L Tris-buffered saline (TBS), pH 7.6. The alkaline phosphatase reaction was revealed by the new Fucsin substrate.

Direct Cytotoxicity Assay

The toxicity of Ber-H2/S06 IT to JB6 cells was evaluated in vitro by a nonradioactive cell cytotoxicity assay (Promega Cell Titer 96; Promega Corp, Madison, WI), based on the cellular conversion of a tetrazolium salt into a blue formazan product that can be detected using an enzyme-linked immunosorbent assay (ELISA) plate reader. Cells, checked for viability and adjusted to a concentration of 10⁵/mL in complete RPMI 1640 medium, were distributed into triplicate wells of a 96-well microtiter plate in 50-μL volume (2 × 10⁶ per well), and 50 μL of Ber-H2/S06 IT solution was added to a final concentration ranging from 5 × 10⁻⁴ to 5 × 10⁻¹⁵ mol/L (as S06). Control samples were run with unconjugated Ber-H2 MoAb, S06 alone, unconjugated Ber-H2 plus S06, and with an anti-CD22/S06 conjugate as a irrelevant IT. The CD30-negative B-cell line LCL was used as an irrelevant target for Ber-H2/S06. Plates were incubated for 24 hours at 37°C in a humidified 5% CO₂ atmosphere, and then 15 μL of dye solution was added to culture wells for 4 hours, followed by an overnight incubation with 100 μL of a solubilization/stop solution. A₅₇₀ was determined using an ELISA plate reader, and the percentage of reduction, compared with untreated control cultures, was used as assessment of the killing. The in vitro toxicity of Ber-H2/S06 IT was also tested in parallel from the inhibition of protein synthesis, as previously described (IC₅₀ = concentration of IT at which the leucine incorporation is inhibited by 50% relative to control culture).

In Vivo Antitumor Experiments

Prevention of tumor development. To evaluate the ability of Ber-H2/S06 in preventing tumor growth, IT therapy was started 24 hours after tumor transplantation. Mice were randomly divided into groups of six to seven, and the Ber-H2/S06 IT was injected IP under sterile conditions in a volume of 200 μL of phosphate-buffered saline (PBS) on days 1, 3, and 5 after transplantation with a daily dose of 3.3 μg as S06 (11.3 μg as Ber-H2/S06). The total dose administered (about 10 μg as S06; 34 μg as Ber-H2/S06) corresponded to 50% of LD₅₀, as established previously. Control mice received S06 alone or the unconjugated MoAb in amounts equivalent to those used in the IT.

Treatment of established subcutaneous tumors. In further experiments, IT therapy was started IP when the tumor had grown SC to approximately 0.5 cm in diameter (day 5 after tumor implantation). On 3 consecutive days, mice received three injections of IT (11.3 μg per mouse per day in 0.2 mL PBS). The total dose of IT was 34 μg (ratio = 10 μg S06:24 μg Ber-H2). Mice injected with PBS, unconjugated Ber-H2 MoAb, or S06 alone, at a dosage matching that in the IT, were used as controls.

Tumor diameters were recorded three times weekly by a caliper,
and tumor volume was estimated, as described above. Experiments were terminated 30 days after the animals were transplanted to keep the tumor diameters below 2 cm. The antitumor effects of different treatments were compared by determining the tumor growth delay, which is the difference between the number of days necessary for tumors in each group to reach 500 mm³ volume as compared with untreated mice.

Histologic and Immunohistologic Analysis of SCID Mice Tissues

To assess possible toxic effects of the IT and changes in tumor phenotype, all animals that developed a tumor in spite of IT treatment were subjected to complete autopsy. Both the tumor and the apparently uninvolved tissues were removed, fixed in 10% neutral buffered formalin or B5, and processed for routine histology.

A portion of the tumor was also snap-frozen in liquid nitrogen for immunohistologic analysis. For this purpose, frozen tissues were cut at 5 μm in a cryostat, air-dried overnight, fixed in acetone for 10 minutes at room temperature, and immunostained by the APAAP technique with the following MoAbs: anti-CD45 (Dako, Glostrup, Denmark); anti-CD30 (Ber-H2; provided by Prof. H. Stein, Free University, Berlin, Germany); anti-IL-2 receptor (CD25, Becton Dickinson, San Jose, CA); and Ki-67.

Establishment and Characterization of Subcultures From Tumor-Bearing Mice

Tumors growing in IT-treated mice were removed under sterile conditions, rinsed in complete RPMI 1640 medium, and finely minced with a scalpel. The tumor fragments were then incubated in complete medium in a 24-well plate for 24 hours and subsequently transferred to 25-mL Falcon tissue culture flasks. When the tumor cells had been reestablished as morphologically homogeneous sublines (usually about 7 to 10 days later), cells were checked for antigen expression by immunocytochemical analysis.

RESULTS

Growth of JB6 in SCID Mice

When transplanted SC into SCID mice, JB6 cells induced the growth of solid tumors in greater than 95% of animals. Tumors reached the diameter of 0.5 cm on average by day 7 (range, 5 to 10 days). Tumor cells showed the typical morphologic features of ALCI, i.e., irregular, often indented nuclear profiles with prominent nucleoli and abundant cytoplasm (Fig 1). The neoplastic cells showed the activated T phenotype of the original JB6 cell line (CD2⁺, CD7⁺, βF1⁺, CD25⁺, CD30⁺, CD71⁺, CDw70⁺; Fig 2). Cytogenetic analysis of tumor-derived cells showed the same chromosome abnormalities (t(2;5)(p23;q35) in all cells examined, confirming the human origin and clonality of the JB6 population.

Characteristics of Ber-H2/S06 IT

Both Ber-H2/S06 and OM-124/S06 conjugates contained an average of 1.7 mol of SO6 per molar of antibody as estimated by the radioactive content and by the protein concentration determined from A₂₅₀. The reactivity pattern of the Ber-H2/S06 IT with normal and pathologic human tissues, as well as with the JB6 cell line (Fig 3), was identical to that expected for the native Ber-H2 in vitro. No reactivity of the Ber-H2/SO6 IT was observed with cytopsins prepared from a variety of CD30-negative human cell lines, as well as with frozen sections from CD30-negative human neoplasms.

In Vitro Cytotoxicity of Ber-H2/S06 IT

The toxicity of Ber-H2/S06 to JB6 cells was assessed by incubating the cells with increasing concentrations of ITs at 37°C for 24 hours. Direct comparison between [H]-leucine incorporation and tetrazolium conversion as methods to assay the cytotoxicity of Ber-H2/SO6 indicated less than a 5% difference in the final results. The Ber-H2/SO6 IT was highly toxic to JB6 cells and inhibited protein synthesis by 50% at concentrations (IC_{50}) of 3.23 × 10⁻¹² mol/L as SO6 (Fig 4). The effects of Ber-H2/SO6 on JB6 as compared with HD-derived cell lines L540 and L428 are summarized in Tables 1 and 2. The cytotoxic effect was specific, as the IT prepared from an anti-CD22 (OM-124) antibody, which does not bind to JB6 cells, was up to 10,000 times less toxic (data not shown). SO6, alone or mixed with the Ber-H2 MoAb (IC_{50}, 5 × 10⁻⁹ mol/L), was more toxic than the irrelevant conjugate (IC_{50}, 5 × 10⁻⁴ mol/L). This finding is likely due to the fact that (1) SO6 loses some activity on irrelevant conjugation, and (2) the conjugate is a larger molecule and presumably enters less easily into cells other than macrophages. The CD30-negative B-cell cell line LCL was resistant to both the conjugate and the Ber-H2.

In Vivo Antitumor Activity of Ber-H2/S06 IT

Prevention of tumor development. A 3-day treatment with nontoxic doses of Ber-H2/S06 IT inhibited the development of JB6 tumors in SCID mice when started 24 hours after transplantation of JB6 tumor. In particular, 23 of 24 untreated control mice developed palpable tumors by day 7 after transplant, whereas only 4 of 19 IT-treated mice developed a tumor that was detectable at day 22 (15 days of delay as compared with untreated mice; Table 3). In contrast, neither unconjugated Ber-H2 nor the toxin alone decreased the incidence of tumor engraftment or induced a delay in tumor growth. Mean time to palpable tumor was 7 ± 1 days in control mice injected with anti-CD30 MoAb or SO6 alone. At day 22 after transplantation, the volume of the subcutaneous mass in the IT-treated mice was markedly smaller as compared with controls (Table 3).

The animals treated with Ber-H2/SO6 and that had no signs of tumor growth after 40 days were either given a further subcutaneous implant of JB6 tumor fragments (six mice) or observed for durability of remission up to 100 days (all other mice). All six animals reimplanted with JB6 developed subcutaneous tumors that grew as fast as an age-matched group of control animals. The IT-treated animals had, therefore, not developed detectable resistance to the tumor cells, suggesting that failures in tumor establishment were not due to an intrinsic mouse-related resistance. None of the animals observed up to 100 days developed tumor.

Treatment of subcutaneous established tumor. We next determined whether treatment with Ber-H2/S06 IT or MoAb alone could induce complete remissions when administered at later stages of tumor growth. Mice received three doses of Ber-H2/SO6 IT on 3 consecutive days, starting when
Fig 1. JB6 tumor in SCID mice (paraffin section). Note the typical morphologic features of ALC1 cells that show irregular, often indented nuclear profiles with prominent nucleoli and abundant cytoplasm. Mitotic figures are readily identified. Stained with hematoxylin & eosin; original magnification (OM), ×1,000.

Fig 2. The same sample as in Fig 1 (frozen section) showing expression of the CD30 molecule by all neoplastic cells (stained in red). APAAP technique, hematoxylin counterstain; OM, ×400.

After 2 months, there was no evidence of disease in 25% of IT-treated animals. Only one of the six responding mice had a relapse 15 days after the end of IT treatment, but the tumor regrowth rate was considerably slower than control growth rates.

Signs of acute IT-related toxicity during the first 7 to 14 days of the study included body weight loss (about 20% at day 5 from the end of therapy), slight transient thrombocytopenia, and increases in serum transaminases. There were no IT-related deaths. No tissue damage was seen at histologic examination of liver, spleen, kidney, lung, and heart peri-

Fig 3. The Ber-H2/S06 IT reacts strongly with the JB6 cell line (cytospin preparation). An identical staining pattern was observed in cell suspensions obtained from JB6 tumors growing in SCID mice. APAAP technique, hematoxylin counterstain; OM, ×1,000.

Fig 4. Protein synthesis inhibition assay on JB6 ALC1-derived cell line. 3H-leucine incorporation experiments are as described in Materials and Methods. Concentration refers to S06. Concentration of antibody is equivalent to that in the IT. Results are mean values of triplicates, with the SD being less than 15%.
Table 1. Characteristics of the Ber-H2/S06 IT

<table>
<thead>
<tr>
<th>Molar ratio S06/Ber-H2</th>
<th>1.7</th>
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</thead>
<tbody>
<tr>
<td>IC50 in CFS</td>
<td>8.18 x 10^{-11} mol/L</td>
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Cytotoxicity assay

<table>
<thead>
<tr>
<th>HD-derived cell line L428</th>
<th>4.90 x 10^{-12} mol/L</th>
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<tbody>
<tr>
<td>HD-derived cell line L540</td>
<td>1.86 x 10^{-12} mol/L</td>
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<tr>
<td>ALCL-derived cell line J86</td>
<td>3.23 x 10^{-12} mol/L</td>
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Antibody binding affinity

<table>
<thead>
<tr>
<th>Antibody binding specificity</th>
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<tbody>
<tr>
<td>80% of native Ber-H2</td>
</tr>
<tr>
<td>None</td>
</tr>
</tbody>
</table>

In vitro toxicity to CD34+ bone marrow cells

| None |

Toxicity to mice (LD50)

| 4 mg/kg (as whole IT)* |

Abbreviation: CFS, cell-free system assay (rabbit reticulocyte lysate).

* By intraperitoneal route.

Table 2. Ber-H2/S06 Inhibition of Protein Synthesis in Vitro

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Ber-H2/S06*</th>
<th>Ber-H2 + S06*</th>
<th>S06</th>
<th>Ber-H2</th>
</tr>
</thead>
<tbody>
<tr>
<td>JB6</td>
<td>3.23 x 10^{-12}</td>
<td>2.14 x 10^{-9}</td>
<td>2.81 x 10^{-9}</td>
<td>&gt;5 x 10^{-9}</td>
</tr>
<tr>
<td>L540</td>
<td>1.86 x 10^{-12}</td>
<td>7.58 x 10^{-9}</td>
<td>3.98 x 10^{-9}</td>
<td>&gt;5 x 10^{-9}</td>
</tr>
<tr>
<td>L428</td>
<td>4.90 x 10^{-12}</td>
<td>7.94 x 10^{-9}</td>
<td>4.57 x 10^{-10}</td>
<td>&gt;5 x 10^{-10}</td>
</tr>
</tbody>
</table>

* As S06
Table 3. Effect of Ber-H2/S06 IT on the Prevention of JB6 Tumor Engraftment

<table>
<thead>
<tr>
<th>Treatment Regimen (daily dose)</th>
<th>No. of Engraftments/No. of Inoculations</th>
<th>Mean Days to Palpable Tumor (± SD)</th>
<th>Mean Tumor Volume by D 22 (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS, 200 µL</td>
<td>23/24</td>
<td>7 ± 1</td>
<td>963 ± 248</td>
</tr>
<tr>
<td>S06, 3.3 µg/200 µL</td>
<td>6/6</td>
<td>7 ± 1</td>
<td>888 ± 426</td>
</tr>
<tr>
<td>Ber-H2, 8 µg/200 µL</td>
<td>6/6</td>
<td>7 ± 1</td>
<td>833 ± 226</td>
</tr>
<tr>
<td>BerH2/S06, 11.3 µg/200 µL</td>
<td>4/19</td>
<td>22 ± 5</td>
<td>49 ± 5 (P = .0001)*</td>
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</table>

Animals received IP injections of IT at days 1, 3, and 5 after tumor transplantation. Control mice were injected with PBS, unconjugated Ber-H2 MoAb, or S06.

* Refers to mice (n = 4) that developed tumors.

reduced or lack of ability to bind the Ber-H2 MoAb is likely to represent one of the most important mechanisms of resistance to treatment. These experimental findings have been recently validated by preliminary phase I clinical trials in leukemias and lymphomas. They seem to explain the relapses observed in IT-treated patients and strongly suggest that ITs directed against the CD30 molecule, as well as other lymphoid-associated antigens (eg, CD19 or CD22), are likely to be most effective when used for treating minimal residual disease in combination with other forms of therapy, such as conventional chemotherapy or autologous bone marrow transplantation. There are two additional reasons to propose such an approach: (1) ITs directed against CD30 as well as other target antigens are expected to kill tumor cells with a mechanism different from that of chemotherapy (eg, inhibition of protein synthesis) and, therefore, are expected to be effective against chemoresistant and/or resting residual neoplastic cells; and (2) chemotherapy- and IT-related toxicities are not overlapping.

In conclusion, the experimental results presented in this study suggest a potential role for the Ber-H2/S06 IT in the therapy of low-burden or minimal residual disease in selected categories of CD30+ ALCL, as well as other lymphoma subtypes (centroblastic, immunoblastic, mediastinal large B-cell, large pleomorphic T-cell, adult T-cell lymphoma leukemia) that might express strongly the CD30 antigen in a variable percentage of cases. In this regard, our JB6/SCID mouse model provides a unique opportunity to further optimize IT treatment strategies including scheduling and dosing and to explore new therapeutic modalities based on the use of anti-CD30 ITs in combination with anti-CD30 radiolabeled antibodies or chemotherapeutic agents.

ACKNOWLEDGMENT

We thank Janet Butmarc, Richard Winpenny, Roberta Pacini, Gisberto Loreti, and Laura Natali Tanci for excellent technical assistance.

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Antitumor activity of anti-CD30 immunotoxin (Ber-H2/saporin) in vitro and in severe combined immunodeficiency disease mice xenografted with human CD30+ anaplastic large-cell lymphoma

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