
By Salvatore Siena, Douglas A. Lappi, Marco Bregni, Anna Formosa, Silvia Villa, Marco Soria, Gianni Bonadonna, and A. Massimo Gianni

The authors conjugated, by a disulphide bond, the antihuman T-lymphocyte (CD5) monoclonal antibody (MoAb) OKT1 to the saporin-6 (SAP) ribosome-inactivating protein of the plant Saponaria officinalis. The resulting OKT1-SAP immunotoxin bound to CD5-expressing target cells and under standard culture conditions specifically suppressed mitogen-induced–T-lymphocyte DNA and protein synthesis in a dose-related manner. T-lymphocyte killing was achieved by five-minute exposure of the target cells to OKT1-SAP. The concentration inhibiting 50% (IC50) of T-lymphocyte DNA synthesis was 0.32 nmol/L. The potency of OKT1-SAP was moderately enhanced by amantadine (IC50 0.08 nmol/L) but not by ammonium chloride or chloroquine. Whole blood components did not interfere with the efficacy of OKT1-SAP, as in vitro treatment of fresh whole blood resulted in effective elimination of clonal peripheral blood T-lymphocytes assessed by a limiting dilution assay. Because these characteristics of T-lymphocyte killing by OKT1-SAP (ie, rapidity of action, potency also without potentiators) and lack of inhibition by whole blood components may be relevant for the use of an immunotoxin as a therapeutic agent in humans, the authors evaluated the stability in vivo and the circulatory clearance of OKT1-SAP in cynomologous monkeys. Following a single intravenous (IV) injection of nontoxic dosages (0.16 to 1.3 mg/kg), an initial rapid decline (t1/2 = 1.0 to 4.1 hours) was followed by a long-lasting slower decrease (t1/2 = 11.6 to 20.6 hours) of OKT1-SAP plasma concentrations as detected by double-antibody solid phase enzyme-linked immunosorbent assay (ELISA) assay. Not only did OKT1-SAP remain intact immunologically but it also retained its biological activity, as measured by the ability of plasma samples from monkeys given immunotoxin to inhibit DNA synthesis in human T-lymphocytes. Taken together the findings presented in this article indicate the feasibility of using OKT1-SAP as a therapeutic tool and provide information that will facilitate the rational use of immunotoxins as a treatment modality in humans.

IMMUNOTOXINS (IT), artificially constructed hybrid molecules composed of cell-specific antibodies coupled to bacterial or plant toxins, represent a new family of immunotherapeutic reagents potentially useful in human transplantation, cancer therapy, and immunoregulation.1 Initial clinical applications of ITs have been described, including the ex vivo treatment of human bone marrow with anti-T-cell ITs prior to transplantation2 and phase-I trials with ricin A-chain ITs as systemic therapeutic agents in patients with graft-versus-host disease (GVHD)3 and malignant diseases.4 In these clinical trials the ability of ricin A-chain ITs to eradicate target cells in vivo has been less dramatic than might have been expected from previous in vitro studies.5 In addition, a series of biological limitations for this category of ITs have been identified. First, after intravenous (IV) administration in animals as well as in humans, ricin A-chain ITs possess a short bioavailability as they are rapidly cleared from the circulation.6 This is mostly due to carbohydrate residues in the ricin A-chain moiety that mediate specific recognition and uptake by liver reticuloendothelial and parenchymal cells.5,6 Second, ricin A-chain ITs, even at saturating dosages in vitro as well as in vivo, exhibit poor cytotoxic activity against target cells. This is most probably due to inactivation by lysosomal proteases.7,8 This problem has been obviated in the ex vivo setting by devising optimal culture conditions at pH 7.8, nonreproducible in vivo, and by adding to the incubation medium adjuvant molecules such as ammonium chloride,9 chloroquine,10 amantadine,11 or monensin,12 which raise the intralysosomal pH, thus preventing the proteolytic inactivation of the IT.13 Based on these studies it is now evident that for the ultimate development of ITs as therapeutic agents useful in vivo, it is necessary to search for new types of ITs with increased resistance to inactivation and with better characteristics of pharmacokinetics.

Saporin-6 (SAP) is a type-1 ribosome-inactivating protein14 or hemitoxin,15 purified from the seeds of the plant Saponaria officinalis.16 SAP differs from the A chain of the ricin or abrin toxin in that it is resistant to proteolytic inactivation, has a highly alkaline isoelectric point (pI > 10), does not contain carbohydrate residues, is not toxic to intact eukaryotic cells,17 and acquires specific cytotoxicity when conjugated to target cell-specific antibodies in a rodent model.18 As these biophysical properties may be relevant to the activity and pharmacokinetics of an IT, the authors conjugated SAP to the antihuman pan–T-lymphocyte (CD5) OKT1 monoclonal antibody (MoAb) OKT1 and studied the in vitro activity of this novel IT, referred to as OKT1-SAP, on human T lymphocytes as well as its in vivo biological stability and pharmacokinetics into a nonhuman primate model. The authors report here that OKT1-SAP induces potent and specific killing of human T lymphocytes under physiologic culture conditions and also without the need of adjuvant.

From the Cristina Gandini Bone Marrow Transplantation Unit, Division of Medical Oncology, Istituto Nazionale Tumori, Milan, Italy; the Laboratory of Molecular Biology, Farmitalia Carlo Erba, Milan, Italy; and the Faculty of Pharmacy, University of Milan, Milan, Italy.

Submitted November 9, 1987; accepted April 22, 1988.

Supported in part by CNR Grant 86-00649-44 to A. Massimo Gianni.

Address reprint requests to Salvatore Siena, MD, Istituto Nazionale Tumori, Via Venezian 1, Milan 20133, Italy.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1988 by Grune & Stratton, Inc.

0006-4971/88/7202-0063$3.00/0

molecules. OKT1-SAP is not inactivated by whole blood components, and once administered IV at nontoxic dosages into monkeys, adequate levels of intact and functional OKT1-SAP are maintained into the circulation long enough to result in the ultimate killing of target cells.

MATERIALS AND METHODS

MoAbs, SAP Ribosome-Inactivating Protein, and Preparation of Immunotoxins

OKT1 and T101 are MoAbs that react with the T1 (CD5) surface antigen, a 65-Kd glycoprotein that is expressed on 90% to 95% of human peripheral blood T lymphocytes, most T-cell-derived hematologic malignancies, chronic B-lymphocytic leukemias,10 and some B cells probably involved in the pathogenesis of autoimmune diseases.11 CD5 antigen is not expressed on human multipotent, myeloid, and erythroid progenitor cells. OKT1 antibody (IgG1) was produced by hybridoma cells (American Type Culture Collection, Rockville, MD) grown as ascites tumors in mice. The antibody was purified by ammonium sulfate precipitation and FPLC chromatography over a mono-S column (Pharmacia Fine Chemicals, Uppsala, Sweden). For the chromatography, antibody was dialyzed v 25 mmol/L sodium citrate, pH 5.0 (buffer A). Buffer B was 0.3 mol/L sodium chloride in buffer A. According to the manufacturer's instructions, chromatography was performed over a mono-S 16/10 with gradients of 0% to 40% A and 0% to 20% B, respectively. The resulting pooled fractions were dialyzed against 0.1 mol/L sodium phosphate, 0.1 mol/L sodium chloride, pH 7.5, and used for conjugation. Purified T101 antibody (IgG2a), gift from Hybritech (San Diego), was used as supplied. OKT1-SAP is not inactivated by whole blood to result in 757 units of activity.

Preparation of Immunotoxins

ANTIHUMAN T-LYMPHOCYTE SAPORIN IMMUNOTOXINS

According to synthesis of ITs. ITs were purified by ion exchange chromatography of the excluded volume. T101 ricin A-chain IT was used to determine the minimum time target cells needed to be exposed to OKT1-SAP to achieve effective cytotoxicity, PBMCs (10^7 cells/mL) were incubated with OKT1-SAP at 37°C in 5% CO₂ humidified atmosphere for various times in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS; Hybri-Sure, Hazleton, Denver, PA), either with or without 1 mmol/L amantadine (1-adamantanamine HCI, Sigma, St. Louis), or 10 mM ammonium chloride (Farmitalia Carlo Erba, Milan, Italy), or 100 µM chloroquine (Sigma). At the end of incubation, after three washings at 4°C, PBMCs were assayed for (1) number of viable cells; (2) inhibition of mitogen-induced DNA synthesis; (3) inhibition of mitogen-induced protein synthesis.

Treatment of Cells with Immunotoxins

Peripheral blood mononuclear cells. Isolated PBMCs (10^7 cells/mL) were incubated with OKT1-SAP at 37°C in 5% CO₂ humidified atmosphere for various times in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS; Hybri-Sure, Hazleton, Denver, PA), either with or without 1 mmol/L amantadine (1-adamantanamine HCI, Sigma, St. Louis), or 10 mM ammonium chloride (Farmitalia Carlo Erba, Milan, Italy), or 100 µM chloroquine (Sigma). At the end of incubation, after three washings at 4°C, PBMCs were assayed for (1) number of viable cells; (2) inhibition of mitogen-induced DNA synthesis; (3) inhibition of mitogen-induced protein synthesis.
were incubated at 37°C with 50 nmol/L OKTI-SAP for two hours
frequency
(1) number of viable cells (Trypan blue dye exclusion test); (2)
assayed for (1) number of viable cells; (2) frequency of clonable T
amantadine.
At the end of incubation, after washings, BM cells
and 2 mmol/L L-glutamine. Triplicate cultures were
stimulated
topoietic progenitor cells
by colony assays.
lymphocytes
by limiting-dilution assay; and (3) frequency of hema-
toepoietic progenitor cells was evaluated by clonal assays as
previously
described.25 T-lymphocyte frequency was determined by
Dilution Assay
Quantitation of Clonable T lymphocytes by Limiting
Dilution Assay
After IT treatment, PBMCs were resuspended in round-bottomed
microplates in 0.2 mL RPMI 1640 supplemented with 15%
FCS, 10 mmol/L HEPES buffer, 100 U/mL penicillin, 100 µg/mL
streptomycin, and 2 mmol/L L-glutamine. Triplicate cultures were
stimulated with 10 µg/mL phytohemagglutinin (PHA-P; Difco,
Detroit), and then incubated at 37°C in a 5% CO2 humidified
atmosphere for 72 hours. To assess the DNA synthesis, cultures were
labeled with 1 µCi/well tritiated thymidine (3H-TdR) eight hours
before harvesting. To assess the protein synthesis, IT-treated-
PHA-stimulated cells were labeled with 1 µCi/well tritiated proline
(3H-Pro) after 48 hours of culture. Cells were harvested using a
Skatron apparatus, and 3H-TdR or 3H-Pro incorporation was quanti-
tified by standard scintillation counting technique.

Quantitation of Clonable T lymphocytes by Limiting
Dilution Assay
After IT treatment the frequency of bone marrow or peripheral
blood T lymphocytes was evaluated by a limiting-dilution microcul-
ture assay in PHA-interleukin (IL)-2-feeder cell-containing
medium, according to Kernan et al.24 with minor modifications as
previously described.23 T-lymphocyte frequency was determined by
the minimum chi-square method from the Poisson distribution
relationship between the cell number seeded per well and the
percentage of negative culture wells.26

Quantitation of Multipotential (CFU-Mix), Erythroid
(BFU-E), and Granulomonocytic (CFU-GM)
Progenitor Cells
The frequency of CFU-Mix, BFU-E, and CFU-GM hematopoietic
progenitor cells was evaluated by clonal assays as previously
described.27

Animals
Cynomologous monkeys (Macaca fascicularis) were housed in a
controlled environment with a 12-hour light/12-hour dark cycle. ITs
were injected as single IV bolus (three to four minutes) injections
into the femoral vein. Blood samples were collected from the
controlateral femoral vein in heparinized syringes. Plasma samples
were stored at 4°C until analyzed within 4 days from collection.
Samples from monkey 34247 (OKT1-SAP 1.3 mg/kg) were ana-
alyzed 1 month after collection. Information about the animals and
details of the OKT1-SAP dosages used in these experiments are
reported in Table 4. Monkey No. 34247 (1.3 mg/kg) received a
mixture of OKT1-SAP (2/3) and T101-SAP (1/3).

Concentration of Intact OKT1-SAP Immunotoxin in
Monkey Blood
The concentration of intact OKT1-SAP was measured by a
solid-phase enzyme-linked immunosorbent assay (ELISA). In this
assay, 96-well microtiter plates were incubated overnight at 4°C
with 40 ng/0.1 mL goat antimouse IgG (Bio-Rad, Richmond, CA).
The wells were then washed and incubated with a blocking solution
(1% bovine serum albumin [BSA] in PBS) for 30 minutes at room
temperature. Blocking solution was removed and standards (0.7 to
5.0 ng) or samples were added to the wells and incubated for one
hour at 37°C. The wells were then washed extensively, and rabbit
anti-SAP,21 linked to horseradish peroxidase, was added at a proper
dilution and incubated one hour at 37°C. Wells were again washed
and color developed using standard methods. Sample optical densi-
ties were compared against a standard curve.

Biological Activity of Circulating OKT1-SAP
Immunotoxin
Serial tenfold dilutions of plasma samples from monkeys given
injections of OKT1-SAP were done with RPMI 1640 containing
15% FCS. Twenty microliters of the undiluted and diluted samples
were added to 180 µL of medium containing 2 x 10^5 PBMCs as a
source of target cells and incubated for two hours at 37°C in 5% CO2
humidified atmosphere. At the end of incubation time cells were
washed and cultured under standard conditions in the presence of
PHA. After 72 hours the DNA synthesis of mitogen-stimulated T
lymphocytes was measured as 3H-TdR incorporation. The extent of
DNA synthesis in the presence of control plasma was taken as
maximal synthesis and was used to calculate the percentage of
inhibition induced by plasma samples from OKT1-SAP-treated
monkeys.

RESULTS

Binding of OKT1-SAP Immunotoxin to Target Cells
OKT1-SAP bound to 65% to 75% of PBMCs, as assessed by
indirect immunofluorescence technique. Thus after cross-
linking SAP to a MoAb, the latter moiety of the conjugate
retained the capability of binding to its respective cell-
surface antigen.

Cytotoxicity of OKT1-SAP Immunotoxin

In an initial series of experiments the effect of OKT1-SAP on
resting human T lymphocytes was evaluated by incubating
PBMCs for two hours with various IT concentrations,
washing them to remove unbound IT, and then activating T
lymphocytes by PHA mitogen. The effect of OKT1-SAP was
estimated by measuring suppression of DNA synthesis during
subsequent mitogen activation. While cell viability assessed by
Trypan blue dye exclusion test at the end of a
two-hour incubation was unaffected. T-lymphocyte DNA
synthesis was suppressed in a dose-related manner (Fig 1,
open symbols). The OKT1-SAP concentration inhibiting 50%
(IC50) of T-lymphocyte DNA synthesis was 0.32 nmol/L.
The IC50 of T101-SAP was 0.30 nmol/L. Under the same
experimental conditions, irrelevant control TEC IgM-SAP
did not inhibit mitogen-induced-T-lymphocyte DNA syn-
thesis. 3H-TdR incorporation was 100 nmol/L TEC IgM-
SAP, 126,573 cpm; 50 nmol/L TEC IgM-SAP, 130,805
cpm; 10 nmol/L TEC IgM-SAP, 128,800 cpm; medium,
127,980 cpm. Equimolar concentrations of unconjugated
SAP and OKT1 antibody had no cytotoxic effect (Fig 1).

Because amantadine, ammonium chloride, and chloro-
quine had been reported to potentiate the in vitro cytotoxicity
of other non-SAP ITs, the authors studied the effect of these
weak bases on the cytotoxic activity of OKT1-SAP and
The reproducibility of the inhibition of T-lymphocyte DNA synthesis by OKT1-SAP was investigated in a series of experiments in which three different preparations were employed. In the absence of potentiators, the $IC_{50}$ of T-lymphocyte DNA synthesis for OKT1-SAP batches DAL-E-21, DAL-E-76, and RG-B-28 were 0.32 nmol/L, 0.30 nmol/L, and 0.34 nmol/L.

By measuring the inhibition of T-lymphocyte DNA synthesis following various incubation times with OKT1-SAP, the authors found that most of the cytotoxic effect of OKT1-SAP was reached by five-minute incubation time (Fig 2). Incubations longer than those showed in Fig 2 (i.e., four hours and 12 hours) did not ameliorate OKT1-SAP cytotoxic potential. Thus an effective IT binding (i.e., leading to cell death) is achieved by a very short exposure of the target cells to OKT1-SAP. After the initial brief exposure the activity of OKT1-SAP plateaus at a different level depending on the presence or absence of amantadine in the incubation medium. In the presence of amantadine, mitogen-induced lymphocyte proliferation is inhibited to less than 10% of controls. This is entirely consistent with what one can expect of an IT recognizing the T1 (CD5) surface antigen that is expressed on 90% to 95% of human peripheral blood T lymphocytes. In the absence of amantadine, mitogen-induced lymphocyte proliferation is inhibited to about 20% of controls and does not increase in spite of incubations beyond five minutes. This may reflect the presence of some cells that in the absence of amantadine are sublethally damaged and remain capable of restoring their proliferative capacity. Whether these cells represent a distinct subpopulation of CD5-negative cells or randomly spared cells remains to be established.

Because internalized SAP primarily interferes with protein synthesis, the authors also investigated the OKT1-SAP effect on T-lymphocyte protein synthesis. Following treatment of PBMCs with OKT1-SAP, the inhibition of mitogen induced-T-lymphocyte $^3$H-TdR incorporation (DNA synthesis) after 72 hours of culture closely correlated with that of $^3$H-Pro incorporation (protein synthesis) after 48 hours of culture. Equimolar concentrations of irrelevant control IgM-SAP, unconjugated SAP, and/or OKT1 antibody did not inhibit either DNA or protein synthesis in PHA-stimulated T lymphocytes (data not shown).

To determine the activity of OKT1-SAP on unseparated human T lymphocytes in whole blood, either medium, unconjugated SAP, or OKT1-SAP were added to heparinized whole peripheral blood immediately after drawing. After

### Table 1. Effect of Weak Bases on the Cytotoxicity of Antihuman Pan-T-cell Immunotoxins Against Human Peripheral Blood T Lymphocytes

<table>
<thead>
<tr>
<th>Immunotoxin (50 nmol/L)</th>
<th>OKT1-SAP</th>
<th>T101-SAP</th>
<th>T101-Ricin A-Chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>15.0 ± 9.1</td>
<td>15.4 ± 7.3</td>
<td>93.0 ± 10.1</td>
</tr>
<tr>
<td>Ammonium chloride (20 mmol/L)</td>
<td>14.7 ± 7.9</td>
<td>18.1 ± 7.3</td>
<td>20.1 ± 3.8</td>
</tr>
<tr>
<td>Chloroquine (0.1 mmol/L)</td>
<td>27.4 ± 4.3</td>
<td>33.2 ± 9.8</td>
<td>33.1 ± 2.8</td>
</tr>
<tr>
<td>Amantadine (1 mmol/L)</td>
<td>6.0 ± 5.1</td>
<td>3.7 ± 5.0</td>
<td>5.6 ± 0.9</td>
</tr>
</tbody>
</table>

Values shown represent the mean ± SD from three separate experiments and are expressed as the percentage of $^3$H-TdR incorporation (DNA synthesis) in immunotoxin-treated samples relative to the medium-treated controls. At tested concentrations, neither ammonium chloride, chloroquine, nor amantadine alone had inhibitory effect on T-lymphocyte DNA synthesis.
incubation, residual T lymphocytes were detected by \( ^{3}H \)-TdR incorporation in PHA-stimulated cultures and by limiting dilution analysis in a PHA-rIL-2-feeder cell-containing assay. A representative of three experiments in which whole blood was incubated with OKT1-SAP for one hour is shown in Fig 3. In panel A the \( ^{3}H \)-TdR incorporation in PHA-stimulated cultures is measured as counts per minute (cpm) and corresponds to 15.9 \( \pm \) 5.1% and 90.4 \( \pm \) 12.5% inhibition of T-lymphocyte proliferation relative to medium-treated controls. Most of the OKT1-SAP cytotoxic effect is achieved by less than ten-minute exposure of the target cells to the immunotoxin.

This result corresponds to a 95.58% depletion of T lymphocytes from whole peripheral blood by OKT1-SAP immunotoxin. Either medium, 20 nmol/L SAP, or 20 nmol/L OKT1-SAP, were added to fresh, unprocessed peripheral blood and incubated at 37°C in 5% CO\(_2\) atmosphere. After one hour mononuclear cells were isolated and assayed for residual T lymphocytes in parallel by \( ^{3}H \)-TdR incorporation (panel A) as well as by limiting dilution analysis (panel B). Note that no enhancer molecule was added to whole blood.

Table 2. Competitive Inhibition of OKT1-SAP Immunotoxin

<table>
<thead>
<tr>
<th>Cytotoxicity by Unconjugated OKT1 Antibody</th>
<th>( ^{3}H )-TdR Incorporation</th>
<th>Percent of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>First Incubation</td>
<td>Second Incubation</td>
<td>Counts per Minute</td>
</tr>
<tr>
<td>Medium</td>
<td>Medium</td>
<td>156,847</td>
</tr>
<tr>
<td>OKT1 (3 ( \mu )mol/L)</td>
<td>Medium</td>
<td>148,793</td>
</tr>
<tr>
<td>OKT11 (3 ( \mu )mol/L)</td>
<td>Medium</td>
<td>150,747</td>
</tr>
<tr>
<td>Medium</td>
<td>OKT1-SAP</td>
<td>9,933</td>
</tr>
<tr>
<td>OKT1 (3 ( \mu )mol/L)</td>
<td>OKT1-SAP</td>
<td>116,716</td>
</tr>
<tr>
<td>OKT1 (0.3 ( \mu )mol/L)</td>
<td>OKT1-SAP</td>
<td>96,933</td>
</tr>
<tr>
<td>OKT1 (0.03 ( \mu )mol/L)</td>
<td>OKT1-SAP</td>
<td>82,768</td>
</tr>
<tr>
<td>OKT11 (3 ( \mu )mol/L)</td>
<td>OKT1-SAP</td>
<td>8,126</td>
</tr>
<tr>
<td>OKT11 (0.3 ( \mu )mol/L)</td>
<td>OKT1-SAP</td>
<td>9,966</td>
</tr>
<tr>
<td>OKT11 (0.03 ( \mu )mol/L)</td>
<td>OKT1-SAP</td>
<td>6,023</td>
</tr>
</tbody>
</table>

PBMCs were first incubated with either medium, OKT1 antibody or OKT11 antibody, washed three times, and then treated for two hours with 50 nmol/L OKT1-SAP immunotoxin plus 1 nmol/L amantadine. Washed cells were then cultured with PHA, and after 72 hours the DNA synthesis was measured as cpm of \( ^{3}H \)-TdR incorporation.
Specific Ex-vivo Depletion of Human Bone Marrow T Lymphocytes by OKT1-SAP Immunotoxin

To determine the feasibility of bone marrow T-cell depletion prior to allogeneic transplantation for GVHD prevention, BM buffy-coat cells were treated with OKT1-SAP in the presence of nontoxic (1 mmol/L) amantadine concentration. After two hours of incubation cells were washed and the frequency of residual clonable T lymphocytes was evaluated by a limiting-dilution assay. As shown in Table 3, a 1.5 to 2.0 log depletion of clonable T lymphocytes was achieved without adversely affecting the recovery of hematopoietic progenitor cells. Irrelevant control TEC IgM-SAP was not toxic to BM hematopoietic progenitors and T lymphocytes. Controlo controls were CFU-Mix, 10.9 ± 0.9 per 2 x 10^5 cells; CFU-GM 118.6 ± 41.8 per 2 x 10^5 cells; BFU-E 98.0 ± 21 per 2 x 10^5 cells. Phenotypic FACS analysis of the cell clones surviving treatment with OKT1-SAP indicated they were mostly (>70%) CD5-negative T lymphocytes. In addition, the fraction of CD5-positive cells escaping OKT1-SAP treatment had a weak intensity of surface CD5 antigen as compared to peripheral blood T lymphocytes (data not shown).

Pharmacokinetics and In Vivo Stability of OKT1-SAP Immunotoxin Into a Primate Model

A semilog plot of OKT1-SAP plasma concentrations following a single IV injection of various nontoxic IT doses in four different monkeys is shown in Fig 4. Data concerning the IT doses administered to each monkey and the circulatory clearance of OKT1-SAP are reported in Table 4. After IT injection, monkeys employed for pharmacokinetics studies did not suffer any clinically apparent toxicity. They all survived in good health longer than 1 year when they were used for other non-IT studies. Toxicology studies were performed in another series of cynomolgous monkeys (1 animal/sex/dose). Following a single IV injection of OKT1-SAP at 0.125, 0.5, and 2.0 mg/kg, tolerance was good in all animals. None to mild and transitory elevation of liver function tests (bilirubin, transaminases, alkaline phosphatase) was noted. Microscopic hematuria was found after 2 mg/kg OKT1-SAP. All animals survived. Day-14 autopsies failed to evidence any macroscopic and microscopic pathologic finding in vital organs including liver (Toxicology Department, Farmitalia Carlo Erba, Italy, data on file).

Plasma levels of OKT1-SAP fitted a two-compartment model of drug distribution (Fig 4). As detailed in Table 4, the α-phase half-lives (t₁/α) of OKT1-SAP into the circulation varied between 1.0 hour and 4.1 hours. The β-phase half-lives (t₁/β) varied between 11.6 hours and 20.6 hours. Intact OKT1-SAP was detectable up to 50 hours even after the administration of the lowest IT dose (0.16 mg/kg).

To learn about the fate of the biological activity of circulating IT, plasma samples from monkeys given injections of OKT1-SAP were checked for their ability to inhibit DNA synthesis in human peripheral blood T lymphocytes. There was a time-dependent decrease in the biological activity (DNA synthesis inhibition) of the IT in the blood that correlated with the immunologic determinations of intact conjugate using ELISA. Serial dilutions of a sample collected six hours after inoculation of 0.65 mg/kg OKT1-SAP inhibited T-lymphocyte DNA synthesis (Fig 5) at a degree that closely correlated to what expected on the basis of the OKT1-SAP level estimated by ELISA on the same sample (8 μg/mL corresponding to 39 nmol/L). Another plasma sample collected 48 hours after the inoculation from the same monkey inhibited mitogen-induced T-lymphocyte DNA synthesis by 85% (1:10), 40% (1:100), and 37% (1:1000). The anti-T-lymphocyte cytotoxic activity of these

Table 3. Specific Ex Vivo Depletion of Human Bone Marrow T lymphocytes by OKT1-SAP Immunotoxin

<table>
<thead>
<tr>
<th></th>
<th>Percentage of Medium-Treated Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Nucleated Cells</td>
</tr>
<tr>
<td></td>
<td>CFU-Mix</td>
</tr>
<tr>
<td>OKT1-SAP</td>
<td>95.7 ± 5.9</td>
</tr>
</tbody>
</table>

Bone marrow buffy-coat cells were incubated for two hours with 50 nmol/L OKT1-SAP plus amantadine as described in "Materials and Methods." Residual T lymphocytes were quantified by a limiting-dilution assay. Values shown are mean ± SD from six (T cells), five (CFU-GM), and one (BFU-E, CFU-Mix) separate experiments.
plasma samples was prevented by preincubating target cells with free OKT1 antibody (data not shown). Thus OKTI-SAP-specific cytotoxicity is maintained intact in vivo.

DISCUSSION

The authors report here that the disulphide linkage of the hemitoxin SAP to the CD5 MoAb OKT1 yields an immunotoxin, referred to as OKTI-SAP, that is specifically cytotoxic to CD5-expressing human T lymphocytes in vitro. Evidence is presented demonstrating that OKTI-SAP possesses distinctive functional characteristics that are profoundly different from those previously reported for other anti-CD5 ITs containing ricin A-chain instead of SAP as the effector toxic moiety. Specifically, the conditions under which T-lymphocyte killing by OKTI-SAP is accomplished imply brief interaction (about ten minutes at 37°C) of the target cells with the IT at a physiologic pH and also without adjuvant molecules artifically added to the culture medium. Furthermore, the authors show the OKTI-SAP is active on fresh whole blood T lymphocytes, thus indicating that blood components do not hamper the efficacy of this IT. In contrast, the authors and other investigators have reported that various anti-CD5 disulphide-linked ricin A-chain ITs are active only under stringent nonphysiologic culture conditions, including the presence of potentiators, at a pH 7.8 after longer incubation times and on isolated cell suspensions without contaminating erythrocytes.7,13,28,29 The different behavior in vitro between ITs with the same specificity but containing different toxins (i.e., anti-CD5 antibodies disulphide linked to either ricin A-chain or SAP) may reflect the biophysical properties of the toxic component of the IT molecule. The hemitoxin SAP is extremely resistant to physical as well as proteolytic inactivation, and it is likely that the peculiar cytotoxicity of OKTI-SAP is due to a slow rate of extracellular and/or intracellular inactivation by intralysosomal proteases.

The cytotoxic activity of other non-SAP ITs is potentiated in vitro by the carboxylic monoamine ammonium chloride, chloroquine, and amantadine.10,13,14 The authors report here that OKTI-SAP cytotoxicity is modestly potentiated only by amantadine and not by ammonium chloride or chloroquine. These findings support the notion that the potentiation of ITs in vitro may vary depending on the type of IT employed and its composition.

Three in vitro functional characteristics of OKTI-SAP (i.e., activity at physiologic culture conditions without potentiators, rapidity of action, and lack of inhibition by whole blood components) prompted us to further investigate the feasibility of using this IT in vivo.

The biological stability of an IT might be the major factor affecting its therapeutic efficacy. The disulphide bond of OKTI-SAP may be disrupted by disulphide exchange with glutathione or other plasma or cell factors. To address the issue of OKTI-SAP stability in a living individual, the authors studied the fate of this IT into a primate model by (1) following the clearance from blood of intact OKTI-SAP by double-antibody–solid-phase ELISA assay, and (2) determining the biological activity of the conjugate by measuring the ability of OKTI-SAP retrieved from the circulation to inhibit DNA synthesis in human peripheral blood T lymphocytes. After single IV bolus injection the clearance of circulating OKTI-SAP shows a biphasic kinetics. An initial rapid decline is followed by a long-lasting slower decrease of OKTI-SAP plasma concentrations. This behavior is commonly exhibited by a variety of drugs and is consistent with an early distribution phase followed by a slow clearance of the IT. The physiologic meaning of this kinetic behavior cannot be addressed on the basis of the presently available data. Evidence is presented demonstrating that OKTI-SAP not only remains immunologically intact but also retains its biological activity and is not subject to degradation that could prevent its cytotoxic action on target cells up to 2 days after administration. The authors also show that an effective OKTI-SAP binding (i.e., leading to cell death) is achieved by a very short exposure of the target cells to this IT. Thus
the presence of immunologically and biologically intact OKT1-SAP into the circulation for a period of over 24 hours would give ample time for selective killing of target cells.

The monkeys employed in our studies do not have the respective CD5-expressing target cells for OKT1-SAP. If target cells were present in the inoculated animals, the actual clearance profile may be different than the observed values. However, the authors report that the length of OKT1-SAP half-lives in the circulation is considerably longer than those previously reported for various disulfide-linked ricin A-chain ITs into nonhuman primate or rodent models. There are several possible explanations for this difference. First, ricin A-chain contains carbohydrate residues, whereas SAP is free from carbohydrates. It has been demonstrated that ricin A-chain ITs are cleared from the bloodstream by liver parenchymal and reticuloendothelial cells that have receptors for the carbohydrates. Chemical deglycosilation of the A-chain greatly reduces liver uptake, thus resulting in an important antitumor effect in spite of residual carbohydrates still contained in the antibody moiety of the deglycosilated A-chain IT. Second, as suggested by Thorpe et al., perhaps the bond between ricin A-chain and the antibody is more labile than that in the SAP conjugate. If so, the better stability of OKT1-SAP could be because SAP (pl > 10) bears positive charge under physiologic conditions, whereas ricin A-chain (pl ~ 7.5) is uncharged. The SAP moiety of the conjugate may therefore interact electrostatically with the negatively charged antibody to form a more compact structure in which the linkage is protected from attack by glutathione. Third, it is possible that the ricin A-chain ITs but not OKT1-SAP, as shown by the authors' results, interacts with blood components to form complexes that are either inactive or rapidly cleared from the animal. The prolonged bioavailability of OKT1-SAP into the circulation may favorably affect the capacity to eradicate target cells and consequently its therapeutic efficacy. If this is the case, OKT1-SAP may prove more effective in vivo than other anti-CD5 ITs containing rich A-chain. In agreement with this possibility, Thorpe et al. found that a single IV injection of anti-Thy1.1 antibody conjugated to SAP into mice bearing a Thy1.1-expressing lymphoma allograft prolonged their survival time by an extent corresponding to that expected if 5 logs of the tumor cells had been eradicated. Interestingly, ricin A-chain coupled to the same antibody was 100- to 1,000-fold less effective than the SAP IT as an agent in vivo, even though the two ITs were equally cytoxic to the lymphoma cells in culture.

An unexpected finding of Thorpe et al. was that the acute toxicity of SAP to mice (LD90 6.8 mg/kg) was elevated eightfold to 16-fold by conjugation to anti-Thy1.1 antibody or to its F(ab')2 fragment. Histologic examination of recipients of the ITs revealed gross damage to hepatic parenchymal cells and to the white pulp of the spleen, neither of which was caused by unconjugated SAP. These findings raised the issue of nonspecific organ toxicity as a major potential limitation for the development of SAP ITs as therapeutic agents. In the authors' experience (Toxicology Department, Farmitalia Carlo Erba, Italy, data on file), the LD90 of a single IV injection of OKT1-SAP in mice (i.e., 6.7 mg/kg in males and 4.3 mg/kg in females) is fourfold to 16-fold higher than that reported by Thorpe et al for their anti-Thy1.1 SAP ITs. In addition, the OKT1-SAP LD90 in rats is even higher than in mice (i.e., 20 mg/kg in males and 11.5 mg/kg in females). These observations together with the pathologic data from the nonhuman primate model (see "Results"), although they do not exclude the potential for nonspecific toxicity by OKT1-SAP in vivo in humans, provide information useful for the clinical application of ITs such as OKT1-SAP in humans.

Several hemitoxins other than SAP have been described. Among these the pokeweed antiviral protein (PAP) from Phytolacca americana conjugated to T-cell-specific antibodies possesses functional characteristics in vitro and in vivo that resemble those of OKT1-SAP. As there are no published data concerning the activity of any anti-CD5 PAP conjugate, a direct comparison with OKT1-SAP is not possible. An advantage of using SAP instead of PAP is the much higher yield of pure hemitoxin from the seeds (0.2% to 0.4%) of Saponaria officinalis as compared to that from the spring leaves (0.01%), summer leaves (0.004%), or seeds (0.1%) of Phytolacca americana.

A clinical application of OKT1-SAP could be its use for the ex vivo depletion of mature T-lymphocytes from bone marrow grafts prior to allogeneic transplantation to prevent GVHD. In 1986 Kernan et al. showed that the number of clonable residual T-lymphocytes measured by a limiting-dilution assay correlated with the development of GVHD in leukemic patients transplanted with histocompatible donor bone marrow T-cell depleted by soybean fractionation and E-rosette depletion. In particular, these authors found that no patients who received less than 1 x 10⁶ residual, clonable bone-marrow T-cells per kilogram of recipient body weight developed GVHD, while among those patients who received between 1 x 10⁷ and 4.4 x 10⁷ T cells per kilogram, about half developed benign grade I-II skin GVHD. Employing the same limiting-dilution assay for quantitation of residual bone marrow T-lymphocytes, the authors show in this article that OKT1-SAP induces a 1.5 to 2.0 log reduction of clonable T-lymphocytes without adversely affecting the recovery of normal hematopoietic cells. Although no data exist demonstrating the clinical value of selective CD5 T-lymphocyte depletion by ITs as sole prophylactic measure for GVHD, based on Kernan's data one can predict that the extent of T-cell depletion achieved by OKT1-SAP may not be sufficient to prevent severe grade III-IV GVHD. If this is the case, OKT1-SAP in association with other means of T-lymphocyte depletion or post-transplant immunosuppression represents a useful reagent for reproducible and specific elimination of mature T-lymphocytes from human bone marrow prior to transplantation in humans.

The authors' data demonstrating the specific cytoxicity, the stability in vivo, and the attainable blood levels of OKT1-SAP immunotoxin suggest that they should be able to deliver intact IT to target T-cells in both the circulation and secondary lymphoid organs. In fact, recent studies in rodents indicate that the authors can achieve specific T-cell ablation in lymph nodes and spleen following IT administration without untoward effects (F. Marcucci et al, manuscript in
REFERENCES


30. Worrel NR, Cumber AJ, Parnell GD, Mirza PA, Forrester


Synthesis and characterization of an antihuman T-lymphocyte saporin immunotoxin (OKT1-SAP) with in vivo stability into nonhuman primates

S Siena, DA Lappi, M Bregni, A Formosa, S Villa, M Soria, G Bonadonna and AM Gianni