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Immunotoxins Containing Recombinant Anti-CTLA-4 Single-Chain Fragment Variable Antibodies and Saporin: In Vitro Results and In Vivo Effects in an Acute Rejection Model

Pier-Luigi Tazzari,* Letizia Polito, † Andrea Bolognesi, 2† Maria-Pia Pistillo, § Paolo Capanni, § Giulio Lelio Palmisano, § Roberto M. Lemoli, § Antonio Curti, § Luigi Biancone, † Giovanni Camussi, † Roberto Conte,* Giovanni B. Ferrara, § and Fiorenzo Stirpe †

Immunotoxins containing recombinant human-derived single-chain fragment variable (scFv) reagents (83 and 40) against CTLA-4 (CD152) linked to saporin, a ribosome-inactivating protein, were prepared and tested on CD3/CD28-activated T lymphocytes, MLRs, CTLA-4-positive cell lines, and hemopoietic precursors. Immunotoxins induced apoptosis in activated T lymphocytes and were able to specifically inhibit MLR between T lymphocytes and dendritic cells. The 83-saporin immunotoxin also inhibited the T cell activation in an MLR between T lymphocytes and an EBV-positive lymphoblastoid B cell line. Toxicity tests on hemopoietic precursors showed little or no effects in inhibiting colonies’ growth. As the 83 scFv Ab was reactive also with activated mouse T lymphocytes, 83-saporin was tested in a model of tumor rejection consisting of C57BL/6 mice bearing a murine H.end endothelioma cell line, derived from DBA/2 mice. The lymphoid infiltration due to the presence of the tumor was reduced to a high extent, demonstrating that the immunotoxin was actually available and active in vivo. Thus, taking the results altogether, this study might represent a new breakthrough for immunotherapy, showing the possibility of targeting CTLA-4 to kill activated T cells, using conjugates containing scFv Abs and type 1 ribosome-inactivating protein. The Journal of Immunology, 2001, 167: 4222–4229.

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3 Abbreviations used in this paper: GvHD, graft-vs-host disease; RIP, ribosome-inactivating protein; AC_{150}, concentration of immunotoxin (expressed as RIP content) causing apoptosis in 50% of the cells; DC, dendritic cell; 2-IT, 2-iminothiolane; scFv, single-chain fragment variable; SPDP, N-succinimidyl-3(2-pyridyldithio) propionylate; BFU, burst-forming unit.
by recombinant techniques. In these molecules, $V_H$ and $V_L$ domains are joined by a peptide linker to form a single fusion protein (20). In our study, two distinct scFvs were used, namely scFv 83 and 40, both recognizing an epitope on the CD152 dimer (18).

**Materials and Methods**

**CTLA-4 scFv Abs isolation**

A human scFv phage library (18, 19) was used to generate cCTLA-4 Abs that were selected and characterized, as already described (18). Specificity was checked by competitive ELISA, Western blot, and immunofluorescence (18). In particular, two scFvs, namely 83 and 40, were further selected on the basis of their reactivity with T cells, both resting and activated (via CD3/CD28) (18). The positivity of the cells was checked by immunofluorescence and analyzed by flow cytometry (EPICS XL; Coulter Immunotech, Hialeah, FL); in particular for scFvs and immunotoxins, reactivity was checked by indirect immunofluorescence, as already described (11, 14, 15, 18). Appropriate negative (mouse IgG; Coulter Immunotech) and positive (CTLA-4 BN13 mAb; Coulter Immunotech) controls were used. The 83 and 40 scFvs were also conjugated to FITC and used for direct staining, following standard procedures. In particular, cell lines were stained by direct immunofluorescence technique, and CD34/4Fc double staining was performed using FITC-conjugated 83 and 40 Abs and a PE-conjugated CD34 (CD34-PE, 581 clone; Coulter Immunotech).

**Immunotoxins**

Type I single-chain RIP saporin S6 (hereupon referred to as saporin) from the seeds of Saponaria officinalis (Azienda Regionale delle Foreste della Regione Emilia Romagna, Casola Valsenio, Ravenna, Italy) was purified essentially as described by Barbieri et al. (21). The RIP was labeled with $^{125}$I with the iodogen reagent (Pierce, Rockford, IL), according to manufacturer’s instructions.

The RIP and the scFv were conjugated via a disulfide bond between chemically inserted sulfhydryl groups. scFvs 83 and 40 were modified using $\text{N}$-succimidyl-3-[2-pyridyl-dithio] propionylamine (SPDP; Sigma, St. Louis, MO) (22) at a 14:1 SPDP:scFv molar ratio. After 30 min at 28 °C, the modified protein was separated from unconjugated reagent by gel filtration on a Sephadex G25 column (30 cm × 1.6 cm) (Pharmacia Biotech, Uppsala, Sweden), equilibrated, and eluted with PBS (0.14 M NaCl in 5 mM sodium phosphate buffer, pH 7.4). The number of sulfhydryl groups introduced was determined by the A$_{343}$ prior to and after reduction with 20 mM 2-ME, as described by Carlsson et al. (23).

Saporin, containing a trace of $^{125}$I-labeled RIP, was dissolved in 50 mM sodium borate buffer, pH 9, at a concentration of 7 mg/ml; the RIP was subsequently modified by adding 2-iminothiolane (2-IT; Sigma) to a final concentration of 1 mM and purified following already published procedures (15). The number of sulfhydryl groups introduced was determined by the A$_{312}$ prior to and after reduction with 20 mM 2-ME, as described by Ellman (24).

The modified RIP was reduced with 20 mM 2-ME, filtered as above through a Sephadex G25 column, and collected directly onto the unreduced derivatized scFv. The RIP/Ab mixture, in a 10:1 molar ratio, was allowed to react for 16 h at room temperature.

The resulting conjugate was separated from the unconjugated reagents and from RIP homopolymers by affinity chromatography with HiTrap rProtein A-Sepharose fast flow column (1 ml; Pharmacia Biotech), equilibrated with PBS, washed with 0.5 M NaCl in 5 mM sodium phosphate buffer, pH 7.4, and eluted with 0.6% glycine in 20 mM HCl, pH 3. Fractions of 1 ml each were collected onto 200 μl of 1 M Tris-HCl, pH 9. The immunotoxin was separated from the unconjugated Ab by gel filtration on a Sephacryl S200 high-resolution column (100 cm × 2.5 cm; Pharmacia Biotech), equilibrated and eluted with PBS. The RIP:Ab ratio of the immunotoxin was estimated by the $^{125}$I-labeled RIP radioactivity and by the protein concentration calculated from the $A_{280}$.

Protein synthesis-inhibitory activity of the pooled fractions was assayed on a rabbit reticulocyte lysate, as described below. The immunotoxins were analyzed by SDS-PAGE with a PhastGel Gradient 10-15 (Pharmacia Biotech) under nonreducing conditions, loading 1 μg of protein in 1 μl sample buffer containing 10 mg/ml iodoacetamide, as described by Lambert et al. (25).

The conjugates were sterilized by filtering through a 0.22-μm filter, divided into aliquots, and stored in liquid nitrogen at 1 μM concentration, as RIP.

**Cell-free protein synthesis inhibition assays**

The inhibitory activity of immunotoxin on cell-free protein synthesis was evaluated with a rabbit reticulocyte lysate, prepared as described by Allen and Schweit (26). Immunotoxins were prior reduced with 20 mM 2-ME for 30 min at 37°C, appropriately diluted, and then added to a reaction mixture, as already described (15, 27). Each experiment was conducted in duplicate. The IC$_{50}$ was calculated by linear regression analysis (15).

**Target cell models**

Immunotoxins were tested in different experimental models, including 1) T cells activated via CD3/CD28; 2) MLR with B lymphoblastoid cell lines as allogeneic target; 3) MLR with monoocyte-derived dendritic cells (DC); and 4) human lymphoid and myeloid cell lines.

**Activated T cells via CD3/CD28**

T cells were isolated from PBL (obtained from healthy random donors after informed consent) either by E-rosetting or by MACS CD3 microbeads and magnetic separation columns (Milenyi Biotec, Bergisch Gladbach, Germany). Purity was always >95%, as checked by direct immunofluorescence with FITC-CD3 and FITC-CD2 mAbs (Coulter Immunotech). T cells were then activated via CD3/CD28 (1 μg/ml each; Coulter Immunotech) in RPMI 1640 medium containing 10% FCS, and checked for surface positivity with mAbs BN13 (anti-CTLA-4 mAb used as positive control) (28) and with FITC 83 and FITC 40. Resting T lymphocytes were used as negative controls. Cells were maintained for 72 h in the presence of immunotoxins, or separate components, mixed or as single reagents.

**MLR with EBV-infected lymphoblastoid B cell line (BM14)**

Standard MLR was performed by using PBL from random healthy donors (after informed consent) and a lymphoblastoid EBV B cell line (BM14) irradiated with 100 Gy in a 1:100 ratio (100,000 effector cells to 1,000 target cells/well in a 96-well round-bottom plate). Cells were cultured for 6 days in a 200-μl final volume of complete medium.

**MLR with monocoyte-derived DC**

DC were generated as follows: mononuclear cells were obtained by gradient centrifugation (Lymphoprep, 1:077 g/ml; Nycomed Pharma, Oslo, Norway) from whole blood of random healthy donors (after informed consent). Light-density cells were washed twice in PBS with 1% BSA (Sigma), and CD14+$^+$ cells were highly purified from mononuclear cell fraction by MiniMacs high-gradient magnetic separation column (Milenyi Biotec), according to the manufacturer’s instructions. Flow cytometry reanalysis of CD14+$^+$ positive cells was performed on a gated population set on scatter properties by using EPICS XL equipment, as earlier described (29). A minimum of 10,000 events was collected in list mode.

DC were obtained from peripheral blood CD14+$^+$ cells, as previously described (29), with some modifications. Briefly, 1 × 10$^6$ purified CD14+$^+$ cells were cultured for 5–7 days in 1 ml of RPMI 1640 supplemented with 10% (Sera Lab, Crawley Down, Sussex, U.K.) fetal calf serum, 25 mM HEPES, 50 mg/ml L-tosylamide, 50 mg/ml L-amino G (Genzyme, Cambridge, MA). When indicated, 25 ng/ml TNF-α (Innogenetics, Zwijndrecht, Belgium) was added to the culture for 24–36 h to induce terminal maturation of DC (29). Cultures were maintained at 37°C in 5% CO$_2$ by replacing culture medium and cytokines at day +3. To check the percentage of DC, dual-color immunofluorescence was performed using the following panel of mAbs: PE or FITC anti-human CD1a (BD PharMingen, San Diego, CA); FITC anti-human CD86 (BD PharMingen); FITC BB1/B7 (anti-CD80; BD Biosciences, San Jose, CA); FITC anti-human HLA-DR (BD Biosciences); FITC or PE Leu-M3 (anti-CD14; BD Biosciences); FITC Leu-4 (anti-CD3; BD Biosciences); PE anti-human CD83 (Coulter Immunotech); FITC anti-human CD40 (BD PharMingen); and FITC-CD4 (anti-CD4; BD Biosciences). Negative controls were isotype-matched irrelevant mAbs (BD Pharmingen and BD Biosciences). Cells were incubated in the dark for 30 min at 4°C in PBS containing 1% BSA. After washing, cells were resuspended in PBS containing 1% paraformaldehyde and analyzed, as reported above.

To test their allogeneic stimulatory activity, DC were irradiated (30 Gy) and tested as stimuli in primary MLR.

**Human cell lines.** Human-derived cell lines were analyzed for surface coexpression of 83 and 40 scFv, and of BN13 anti-CTLA-4 reference mAb (28). The following cell lines were evaluated by immunofluorescence: Daudi and Raji, derived from a Burkitt lymphoma; CEM, Jurkat, and Molt 4, derived from T lymphoblastic cells; L428, a B cell line derived from a Hodgkin’s lymphoma; HOM-2, a B lymphoblastoid cell line; K562, an erythroleukemia cell line; HL60, a myeloid-leukemia-derived cell line. Positive cells were used to test the toxicity of immunotoxins, as described below.

Moreover, RNA analysis of CTLA-4 extracellular domain was compared in HL60, K562, and HOM-2 and in resting and CD3/CD28-activated
PBLs. The RT-PCR was performed using specific primers (forward primer, 5'-ATGCACGTGGGGCACTGCTG-3' ; reverse primer, 5'-TCTAGA CCGCTGCAACAGGACCT-3' ) (30). Total RNA was prepared using 500 μl of RNA clean reagent (Hybaid-AGS, Heidelberg, Germany) for 2 × 10^6 cells. The obtained RNA was quality checked by electrophoresis in a 1% agarose gel; cDNA was prepared from 5 μg of total RNA with a reverse-transcriptase mixture (500 ng of RNA). Notably, the PCR conditions were designed to maintain the reaction efficiency in logarithmic phase. The intensity of electrophoretic bands with background subtraction was measured in a Bio-Rad densitometer (type Gel Doc 1000; Bio-Rad, Hercules, CA), and the adjusted volume was calculated for each band. All specific PCR products were confirmed by direct sequence analysis with an automated sequencer (ABI PRISM 377; PerkinElmer, Santa Clara, CA).

Toxicity tests

MLRs. Effector T cells and target DC (100:1) were resuspended in RPMI 1640, 25 mM HEPES, antibiotics, and 15% AB human serum that had been inactivated at 56°C for 30 min. Allogeneic T cells (5 × 10^6) were mixed with stimulators (DC) in round-bottom 96-well plates for 6 days at 37°C in a 5% CO2 humidified atmosphere, as described above for MLR with EBV-infected lymphoblastoid B cells (BM14), in the presence of immunotoxins, or separated components, mixed or single.

Cells were pulsed with 1 μCi/well [3H]ThDUR (Pharmacia Biotech) on day 6, and after another 18 h, cells were harvested with an automatic cell harvester (Skatron Instruments, Lier, Norway) onto glass-fiber diskettes. The radioactivity incorporated was measured with a beta counter (Beckman, Fullerton, CA). Each experiment, performed three times, was conducted in triplicates. IC50 was calculated by linear regression analysis.

Inhibition of protein synthesis in activated T lymphocytes and cell lines. The effects of the immunotoxins on CD3/CD28-activated T lymphocytes and on cell lines were evaluated from the inhibition of [3H]leucine incorporation. Cells were harvested, checked for viability, and adjusted to a concentration of 10^6 cells/ml in complete RPMI 1640 medium. Cells (10^5 well) were seeded in 96-well microtiter plates (Falcon; BD Biosciences) in a 100-μl volume, and 100 μl of immunotoxin was added to find final concentrations ranging from 10^-5 to 10^-7 M, as RIP. Parallel samples were run with RIP alone, anti-CTLA-4/Ab and RIP, and a mixture of unconjugated Ab and RIP. After 72 h, 74 kBq/μl [3H]leucine (Pharmacia Biotech) was added, and after another 18 h, cells were harvested with an automatic cell harvester (Skatron Instruments) onto glass-fiber diskettes. The radioactivity incorporated was measured with a beta counter (Beckman). Each experiment, performed three times, was conducted in triplicate. IC50 was calculated by linear regression analysis.

Evaluation of apoptosis by FITC-Annexin V binding and propidium iodide staining. In a series of experiments performed on CD3/CD28-stimulated lymphocytes or a cell line (HOM-2), viable and dead cells were evaluated by a double staining via FITC Annexin V and propidium iodide with a kit purchased from Bender Medsystem (Wien, Austria), following manufacturer’s instructions. Cells incubated with different concentrations of immunotoxins, RIP, scFv Abs, or a mix of RIP and scFv Abs were cultured in complete RPMI 1640 medium for 72 h. Analysis was performed by flow cytometry, calculating both the percentage of apoptotic (annexin V+/propidium iodide−) and necrotic (annexin V+/propidium iodide+) cells. The concentration of immunotoxin causing apoptosis in 50% of the cells (AC50) was calculated by linear regression analysis.

Toxicity to hematopoietic precursors. Tests were performed using CD34+ cells purified from normal donors, as already described (31). Briefly, mononuclear cells were separated onto Ficoll-Hypaque gradient (Lympho- flott, Biotest, Dreieich, Germany). CD34+ cells were then purified with MACS CD34+ microbeads and magnetic separation columns. Purity was assessed by flow cytometry with a CD34-FITC mAb (Coulter Immunotech) and propidium iodide dye exclusion for dead cells. For colony assay, 5 × 10^3 cells were seeded in semisolid medium consisting of 1 ml of IMDM supplemented with 24% FCS, 0.8% BSA, 10^-4 M 2-ME, 2 μl of human recombinant erythropoietin (Dompé Biotec, Milan, Italy), and 2 × 10^-4 M bovine hemin. To measure the optimum clonogenic efficiency, 10% (v/v) of a selected batch of a PHA-lymphocyte-conditioned medium was added. SCFv 83-saporin conjugate, scFv 83, saporin, or a mixture of the two was added to the cultures at 10^-5 and 10^-6 M concentrations. Methylcellulose final concentration was 1.1%. Granulocyte-macrophage CFU and burst-forming unit (BFU) erythroid were scored after 14 days of incubation at 37°C in a fully humidified 5% CO2 atmosphere. The clonogenic efficiency of CD34+ cells was 7.3 ± 3.1%.

Mouse in vivo model. To preliminarily verify the in vivo availability and activity of the conjugate, we used a mouse model consisting of implanted allogeneic cells, capable of giving rise to an acute immune rejection reaction with a T lymphoid infiltrate, as described by Garlanda et al. (32). Briefly, cells from the murine H.end endothelioma, able to induce a tumor when implanted s.c., were derived from DBA/2 mice. These cells are able to elicit an allogeneic response when injected in C57BL/6 mice, with a picture comparable with an acute rejection showing a T lymphoid cell inflammatory infiltrate. The endothelioma cells are quickly killed, while an immune suppression could diminish the reaction and prolong the survival of grafted cells.

To set the model, these cells were cultured in DMEM ( Irvine Scientific, Santa Ana, CA), supplemented with 2 mM glutamine (Life Technologies, Paisley, U.K.), 10% FCS (Irvine Scientific), and gentamicin, before the in vivo injection in C57BL/6 mice. Moreover, C57BL/6 PHA/PMMA-activated T lymphocytes were matched with FITC 83 scFv. The reagent stained the activated T lymphocytes, thus indicating that 83-saporin immunotoxin effectively could target in vivo the activated mice lymphocytes infiltrating the allogeneic tumor.

For in vivo experiments, cells were gently detached from plates with EDTA, washed with PBS, counted in a microcytometer chamber, and resuspended in saline. Cells (10^7) in a total volume of 150 μl were injected s.c. into the left back of 8-wk-old female C57BL/6 mice using a 1-ml syringe with a 26-gauge needle at day 0. A group of four mice was injected i.p. at days 0, 1, and 2, with 4 μg of anti-CTLA-4 immunotoxin 83-saporin, diluted in 100 μl of PBS. As a control, four mice were injected with PBS alone with the same schedule. Animals were sacrificed at day 3 and subjected to autopsy, because systemic acute toxicity usually appears within 48 h. Tissue corresponding to the site of injection was fixed in 10% form-aldehyde and processed for light microscopy. Ten fields were examined at magnification ×250, and infiltrating lymphoid cells were counted by two different investigators.

Results

Immunotoxins

Two immunotoxins were prepared with the anti-CTLA-4 scFvs 83 and 40, conjugated to the type 1 RIP saporin by an artificial disulfide bond. Sulhydryl groups were inserted in the saporin by an imidoester reaction between 2-IT and the primary amino groups of the proteins, as previously reported (15). One thiol group per molecule was inserted in saporin, by using a 4-fold molar excess of 2-IT (Table I).

Because the 2-IT did not react with scFvs in standard conditions, the more reactive linking reagent SPDP was used for these molecules. The scFvs showed a low reactivity also with the SPDP, but a range of 0.78–1.15 thiol groups was inserted per molecule, using

| Table I. Characteristics of the derivatized Ab and RIP, and of the immunotoxins |
|-----------------------------------|-----------------|-----------------|-----------------|-----------------|
|                                  | Free Ab          | Free RIP        | Immunotoxin     |
|                                  | (mol/mol)        | (mol/mol)       | (mol/mol)       |
|                                  | Thiol groups     | Thiol groups    | IC50a            |
|                                  | inserted         | inserted         | (μg/ml)         |
|                                  | (mol/mol)        | (μM)            | (μg/ml)         |
| Spdp added                       | 0.14             | 0.11             | 0.97             | 0.78             |
| IC50b                           | 0.14             | 0.11             | 0.97             | 0.78             |

The IC50 of protein synthesis in a rabbit reticulocyte lysate system was calculated by linear regression analysis.

The IC50 of the immunotoxin is referred to as the RIP content.
a 14-fold molar excess of reagent. After conjugation, the toxin/Ab molar ratio was 1, approximately (Table I). The inhibitory activity of native and conjugated RIP on protein synthesis by a rabbit reticulocytes lysate is also reported in Table I. Despite a loss of activity of 3- to 5-fold, saporin still retained a high protein synthesis-inhibitory activity after conjugation.

**Reactivity of immunotoxins**

To evaluate the possible change in reactivity of the conjugates, indirect immunofluorescence was performed and the degree of cell staining was analyzed by flow cytometry. The scFv Abs after conjugation with saporin maintained their reactivity with T cells activated via CD3/CD28. All tested cell lines reacted with BN13 anti-CTLA-4 reference mAb (28) and were found positive for the 83-recognized epitope, with different degrees of reactivity (Table II). All cell lines showed a weaker staining in comparison with activated (via CD3/CD28) T cells. To confirm and extend these results, we performed the RT-PCR on three representative cell lines (HL60, K562, HOM-2) and on resting and activated PBLs. The transcript of the CTLA-4 extracellular domain was present both in cell lines and in activated PBLs. The intensity of electrophoretic band was 28.0% (HL60), 26.1% (K562), and 32.6% (HOM-2) compared with activated PBLs. Resting PBLs were negative.

All cells were used as target for the 83-saporin immunotoxin. As described in previous studies for scFv 83 and 40 (18), 83-saporin showed a stronger reactivity in comparison with 40-saporin immunotoxin on activated T lymphocytes, thus confirming the recognition of a different epitope (18).

**FIGURE 1.** Flow cytometric analysis of CD3/CD28-stimulated human lymphocytes stained via FITC annexin V and propidium iodide. A, FACS analysis of CD3/CD28-stimulated lymphocytes not treated (CTR) and treated for 72 h with 10^{-10} M 83-saporin immunotoxin and of unstimulated lymphocytes incubated with 10^{-10} M 83-saporin. Regions for annexin V positive only (annexin V+/propidium iodide−) are *upper left*, and those double positive (annexin V+/propidium iodide+) are *upper right*. B, Percentage of viable CD3/CD28-stimulated lymphocytes treated for 72 h with scalar doses of immunotoxin (●), saporin (●), scFv (■), or a mixture of saporin and Ab (▲). The percentage of apoptosis in unstimulated lymphocytes is also reported (★). Results are means of three different experiments. SD never exceeded 10%.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Negative control</th>
<th>BN13 mAb</th>
<th>#83 scFv</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activated T lymphocytes</td>
<td>1.2</td>
<td>11.7</td>
<td>13.1</td>
</tr>
<tr>
<td>Daudi</td>
<td>0.9</td>
<td>4.2</td>
<td>5.6</td>
</tr>
<tr>
<td>Raji</td>
<td>1.2</td>
<td>4.7</td>
<td>5.1</td>
</tr>
<tr>
<td>CEM</td>
<td>1.1</td>
<td>3.7</td>
<td>4.8</td>
</tr>
<tr>
<td>Jurkat</td>
<td>1.3</td>
<td>4.2</td>
<td>4.0</td>
</tr>
<tr>
<td>Molt 4</td>
<td>1.2</td>
<td>8.2</td>
<td>9.7</td>
</tr>
<tr>
<td>HL60</td>
<td>0.9</td>
<td>7.8</td>
<td>9.5</td>
</tr>
<tr>
<td>L428</td>
<td>1.0</td>
<td>7.1</td>
<td>8.7</td>
</tr>
<tr>
<td>HOM-2</td>
<td>1.0</td>
<td>8.1</td>
<td>9.0</td>
</tr>
<tr>
<td>K562</td>
<td>1.1</td>
<td>7.9</td>
<td>8.9</td>
</tr>
</tbody>
</table>
Cytotoxicity tests

In previous studies, we demonstrated that RIP-containing conjugates kill cells via apoptosis (15, 27). Thus, to ascertain the effectiveness of immunotoxins in killing cells, we evaluated the apoptotic phenomenon on target cells, using the annexin V/propidium iodide staining. A series of tests were performed using CD3/CD28-stimulated T lymphocytes, expressing CTLA-4 Ag. Both 83- and 40-saporin immunotoxins were specifically toxic to activated T cells. In particular, 83-saporin had an AC₅₀ of 2.33 × 10⁻¹² M, as saporin, on CD3/CD28-stimulated T lymphocytes, while 40-saporin had an AC₅₀ of 4.29 × 10⁻¹¹ M (Fig. 1). Saporin alone or mixed with scFvs was weakly toxic only at 10⁻⁸ M concentration. Unstimulated T lymphocytes were not affected (Fig. 1). The 83-saporin caused apoptosis in >90% of CD3/CD28-stimulated T lymphocytes, at concentrations ≥10⁻¹⁰ M. The most effective immunotoxin (83-saporin), tested either on a model of CD3/CD28-stimulated lymphocytes incorporating [³H]leucine or in an MLR having an EBV-infected B lymphoblastoid cell line as target, had an IC₅₀ of 7.83 × 10⁻¹¹ M and of 7.20 × 10⁻¹¹ M as saporin, respectively, whereas a mixture of unconjugated scFv and RIP had a limited toxicity only at 10⁻⁸ M.

A possible application of these immunotoxins might be the in vivo therapy of transplanted organ rejection and GVHD. Thus, to test the efficacy of these immunotoxins, we designed a model of alloreactivity, performing a series of tests using T cells as effectors and DC as stimulators. These tests showed a high efficiency of the immunotoxins in stopping the proliferation of T lymphocytes, [³H]TdR incorporation being inhibited by 83-saporin with an IC₅₀ of 3.68 × 10⁻¹⁰ M as saporin and by 40-saporin with an IC₅₀ of

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### Table III. Inhibitory effect of immunotoxin and a mixture of free saporin and #83 on protein synthesis by target cell lines

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>S06/#83 IC₅₀ (nM)</th>
<th>SO6 + #83 IC₅₀ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daudi</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Raji</td>
<td>11.8</td>
<td>50.0</td>
</tr>
<tr>
<td>CEM</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Jurkat</td>
<td>15.0</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Molt 4</td>
<td>5.18</td>
<td>37.0</td>
</tr>
<tr>
<td>HL60</td>
<td>1.2</td>
<td>7.71</td>
</tr>
<tr>
<td>L428</td>
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</tr>
<tr>
<td>HOM-2</td>
<td>0.13</td>
<td>36.5</td>
</tr>
<tr>
<td>K562</td>
<td>3.36</td>
<td>49.0</td>
</tr>
</tbody>
</table>

*Data are expressed as means of three separate experiments, each performed in triplicate (SD < 15%).

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**FIGURE 2.** Effect of immunotoxins on the proliferation of MLR-stimulated lymphocytes. Allogeneic T cells were mixed with irradiated DC, used as stimulator in primary, MLR, and incubated with immunotoxin (●), saporin (○), scFv (■), or a mixture of saporin and scFv (▲). After 6 days and another 18 h with [³H]thymidine, cell proliferation was measured. Results are means of three different experiments. SD never exceeded 10%.

**FIGURE 3.** Toxicity of 83-saporin immunotoxin on HOM-2 cell line. A. Protein synthesis inhibition assay on cells treated with 83-saporin (●) or a mixture of free saporin and 83 scFv (○). After 72 h of incubation and another 18 h with [³H]leucine, the radioactivity incorporated was determined. B. Percentage of viable cells stained via FITC Annexin V and propidium iodide. HOM-2 cells were treated for 72 h with 83-saporin (●) or a mixture of saporin and 83 scFv (○). Results are means of three different experiments, each performed in triplicate. SD never exceeded 10%.
7.17 × 10^{-10} M as saporin, whereas control substances had no effect (Fig. 2).

We explored also the possibility of killing cells constitutively expressing CTLA-4 epitopes recognized by 83 scFv, because the related immunotoxin was shown to be the most effective in comparison with 40-saporin conjugate. All the tested cell lines were positive for 83 and anti-CTLA-4 BN13 mAb, thus being potential targets for in vitro studies. The HOM-2 was the most sensitive cell line (Table III) with an IC_{50} of 1.3 × 10^{-10} M (Fig. 3A). These cells underwent apoptosis with an AC_{50} of 2.79 × 10^{-10} M, and were 100% apoptotic at 10^{-8} M immunotoxin concentration (Fig. 3B), while controls incubated with 83 + saporin were not affected. The toxicity of the immunotoxin, as compared with that of the mixture of scFv and saporin, was not detectable in the tested concentration range both on Daudi and CEM cell lines.

Table IV. Rescue of bone marrow CFU after exposure to immunotoxin, RIP, #83, or a mixture of both unconjugated reagents

<table>
<thead>
<tr>
<th>CFU-GM (% of controls)</th>
<th>BFU-e (% of controls)</th>
</tr>
</thead>
<tbody>
<tr>
<td>#83-Saporin</td>
<td></td>
</tr>
<tr>
<td>10^{-8} M</td>
<td>85</td>
</tr>
<tr>
<td>10^{-9} M</td>
<td>109</td>
</tr>
<tr>
<td>Saporin</td>
<td></td>
</tr>
<tr>
<td>10^{-8} M</td>
<td>88</td>
</tr>
<tr>
<td>10^{-9} M</td>
<td>112</td>
</tr>
<tr>
<td>#83</td>
<td></td>
</tr>
<tr>
<td>10^{-8} M</td>
<td>99</td>
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<tr>
<td>10^{-9} M</td>
<td>100</td>
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<tr>
<td>#83 + Saporin</td>
<td></td>
</tr>
<tr>
<td>10^{-8} M</td>
<td>85</td>
</tr>
<tr>
<td>10^{-9} M</td>
<td>98</td>
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</tbody>
</table>

* Results are given of three separate experiments, each performed in duplicate. Data are mean percentage of control values which were 234 ± 32 (CFU-GM) and 326 ± 101 (BFU-erythroid). SD never exceeded 30%.

Bone marrow precursors assay

Toxicity of 83-saporin was analyzed on CFU obtained from normal G-CSF-mobilized peripheral blood stem cell (Table IV). The immunotoxin appeared slightly toxic on granulocyte-macrophage CFU and BFU erythroid only at the highest concentration tested (10^{-8} M). Same results were obtained with free saporin, alone or mixed with the Ab, whereas 83 scFv was not toxic.

Mouse in vivo model

H.end cells implanted in allogeneic mice typically form highly vascularized tumors characterized by massive lymphocyte infiltration with rapid destruction of the implant, showing a picture largely overlapping that of an acute rejection. It has been shown that this event is strictly T cell dependent because it does not occur in athymic mice (32). As shown in Fig. 4, A and C, a marked infiltration of lymphocytes was observed in H.end implants in allogeneic mice after control treatment with PBS with a mean SD of 256 ± 54 in infiltrating lymphoid cells. In contrast, scarce to moderate infiltration of inflammatory cells was detectable within the tumor of mice treated with 12 μg (total dose) of 83-saporin immunotoxin with a mean SD of 69 ± 42 cells (Fig. 4, B and D). It should be highlighted that, in treated mice, no lymphoid-infiltrating cells were detectable in many fields, whereas allogeneic tumor cells were completely spared. At postmortem examination, no signs of acute organ toxicity were observed. These results demonstrated the actual in vivo availability of the 83-saporin immunotoxin and the ability of killing activated T cells of the host, thus prolonging the survival of allogeneic implanted cells. Moreover, this model may provide evidence that an acute immune organ rejection could be prevented, or possibly reversed, with an appropriate immunotherapy able to kill activated T lymphocytes.

Discussion

Immunotoxins are chemical tools containing toxic moieties intended for the killing of selected populations. These reagents have been devised mainly for the in vivo and ex vivo therapy of tumors.

FIGURE 4. Histological (H&E) analysis of tumors from mice injected with H.end cells and treated with PBS (A and C) or anti-CTLA-4 immunotoxin (B and D). A and C, A marked lymphoid infiltration is observed (A, ×100; C, ×400). B and D, Micrographs showing aspects representative of H.end implant with scarce or absent lymphoid infiltration (B, ×100; D, ×400).
(8, 33, 34), as well as for the in vivo killing of activated T cells responsible for organ rejection, for GVHD, and for autoimmune disorders (10, 31, 35).

In a previous study, a series of CD80/86 immunotoxins were described that could kill CD80/86-positive cells, these Ags being present on APCs (15). To better control the interactions between activated T cells and APC, it could be useful to construct conjugates able to selectively target T cells recognizing APC. CTLA-4 is an Ag that is specifically expressed on the surface of T cells during APC recognition. Thus, a selective killing of activated T cells during the early phases of alloimmune stem cell transplantation or organ transplantation might help in preventing GVHD and graft rejection.

In addition, it is possible to use scFvs as carriers of toxic moieties. In fact, the use of small human Ab fragments as toxin vehicles will have the advantages of a lower immunogenicity compared with xenogeneic Abs, a better tissue penetration, and nonaccumulation in the liver. The lack of C regions would mitigate retention by Fc receptors, found in most tissues and organs, thus further reducing their possible side effects and the probability of giving rise to an immune response due to a low degree of Ag internalization in APC.

In this view, we designed and synthesized by chemical link two immunotoxins containing two different scFvs (83 and 40) against CTLA-4 and the type 1 RIP saporin, which is one of the most effective toxic moieties used to generate immunotoxins. The conjugates were able to selectively kill T cells, activated via CD3/CD28, as well as to inhibit MLRs obtained by mixing T cells and allogeneic APC (in vitro generated DC). Moreover, 83-saporin could inhibit the proliferation of T cells when EBV-positive B lymphoblastoid cells were used as target for the MLR. We studied also the capacity of the 83-saporin immunotoxin of killing cells constitutively expressing CTLA-4. Although results varied depending on the cell line, probably due to a different recycling or internalization of the recognized Ag, we demonstrated that at least six cell lines were killed in a selective fashion, in comparison with control experiments. In fact, HOM-2, K562, L428, HL60, Jurkat, and Molt-4 cells showed a significant sensitivity to the action of 83-saporin conjugate, in comparison with the unconjugated components.

Toxicity to hemopoietic precursors of 83-saporin, the most efficient immunotoxin both on activated lymphocytes and cell lines, was nearly absent, or was present only at a low degree, at the highest dose tested (10−8 M). The latter results indicate that a possible in vitro use of the immunotoxin should not prevent the engraftment of the transplanted hemopoietic stem cells, and that the in vivo administration should not be limited by bone marrow toxicity.

Fusion proteins containing a type 1 RIP have been prepared with human growth factors or hormones (36–40), and with a humanized CDS (41). In the present study, for the first time conjugates containing human-derived recombinant scFvs and a type 1 RIP (saporin) are described.

As to the use of scFvs, it should be highlighted that they might be too small to sterically occupy the recognized epitope and to give any trigger signal, thus being suitable to carry modifier molecules. In this instance, we obtained cytotoxic conjugates, but it is possible to obtain conjugates with drugs, biological response modifiers, and other Abs, the latter for a targeting with bispecific Abs.

Anti-CTLA-4 Abs linked to toxic moieties might be a new tool to prevent acute GVHD and rejection. Maximal expression of CTLA-4 occurs within 48–72 h after T cell activation. A cytotoxic conjugate, which could selectively kill activated T cells involved in GVHD or in graft rejection, if given in the early phases after transplant, might ameliorate the clinical outcome. Our results showed that both conjugates efficiently prevent the proliferation of T cells activated by DC, one of the most potent stimuli to activate alloimmune T cell responders.

A preliminary study, performed in a mouse model of allogeneic reaction, indicates that the 83-saporin actually could well target and kill activated T lymphocytes, thus reducing the lymphoid infiltration and prolonging the survival of the allogeneic cells. Although unusual, the herein described model strongly overlapped the events occurring in a transplanted organ rejection, because an allogeneic endothelial cell population elicits an immune acute rejection mediated by T lymphocytes (32). These results strongly support the view that the 83-saporin conjugate could be used to eliminate activated T cells, which are early basic events in organ rejection and in GVHD. As a consequence, the conjugate could be used in the early posttransplant follow-up within the immunosuppressive therapy schedule. Moreover, this latter experiment could open the way to a clinical approach for targeting with scFv-RIP conjugates.

The targeting of CTLA-4 with scFv-saporin conjugates might not be limited to GVHD or graft rejection, but it is possible to conceive an ex vivo or an in vivo model for the therapy of CTLA-4-bearing tumors. The presence of CTLA-4 on malignant (42) and stimulated normal B cells (43, 44) was previously demonstrated. During our tests, we found that CTLA-4 epitopes are constitutively expressed by some cell lines and myeloid and lymphoid leukemia cells. These findings could open new insights in CTLA-4 expression patterns and lead to a possible application for neoplastic cell depletion. The cytotoxicity results showed that a preclinical test should be performed anyway, because there is a great variability in the sensitivity of tumor cells to anti-CTLA-4 immunotoxins.

A possible concern might be raised about the fact that both in vivo and in vitro small percentages of T cells might escape the immunotoxin, being spared and thus being able to repopulate. It will be possible to control a T lymphocyte repopulation, after the acute activation phase, by using different immunotoxins containing scFv recognizing other epitopes or Ag, as well as protocols for immunosuppression combining the action of immunotoxins and drugs.

Finally, experimental in vivo kinetics studies will be required to verify a possible faster clearance of scFv-containing conjugates in comparison with conventional immunotoxins containing the whole IgG molecule. It should be highlighted that the molecular mass of the conjugates in the present study is ~60 kDa (compared with 66 kDa of albumin), thus probably avoiding the excessive renal clearance of the scFv alone, which has a molecular mass of 30 kDa.

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References


