Cancer Immunol Immunother (1988) 26: 231–236

Cancer Immunology Immunotherapy

An immunotoxin containing a rat IgM monoclonal antibody (Campath 1) and saporin 6: effect on T lymphocytes and hemopoietic cells*

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Summary. The elimination of the cells responsible for graft-versus-host disease in allogeneic bone marrow transplantation has been attempted with a variety of methods, including the use of the ribosome-inactivating toxin ricin bound to monoclonal antibodies acting as carriers. However the high nonspecific toxicity of these immunotoxins containing the whole toxin greatly limited clinical application. Toxicity can be reduced using the A-chain of ricin or other ribosome-inactivating proteins (RIPs) which are devoid of a B-chain with lectin properties. We used saporin 6 purified from *Saponaria officinalis* seeds, which was conjugated with the rat IgM monoclonal antibody Campath 1 specific for mature T and B lymphocytes as well as for monocytes. The immunotoxin retained both RIP and antibody activity, inhibiting protein synthesis both in a cell-free system and in cells bearing the Campath 1 antigen; it also abolished methyl ^{3}H-thymidine uptake in phytohemagglutinin-stimulated T lymphocytes. Myeloid progenitors were largely spared as shown by myeloid stem cell (CFU-GM) growth which was scarcely affected. Toxicity of the immunotoxin to cell lines not expressing the antigen recognized by Campath 1 monoclonal antibody was not greater than the toxicity due to free saporin 6, while the immunotoxin was more toxic to mice than free saporin.

Introduction

A variety of immunological and pharmacological strategies are currently being evaluated for the ex vivo treatment of bone marrow in order to avoid (1) graft-versus-host disease (GVHD) in patients undergoing allogeneic bone marrow transplantation (BMT) [28, 29] and (2) relapses in patients treated with autologous BMT for hematological malignancies [9]. Several methods are currently under development [4, 13, 25]. Many of them are based on the complement-mediated cell lysis obtained by specific monoclonal antibodies (MoAbs) [29]. Large clinical experience has accumulated in the prevention of GVHD with the rat MoAb Campath 1. This IgM antibody recognizes T and B lymphocytes, monocytes, and fixes human complement [2, 16, 20].

A number of studies have reported the possible use of immunotoxins in the prevention of GVHD [49]. The most frequently used toxin is ricin, the toxin of *Ricinus communis* [12, 27, 36, 40, 48]. The B-chain subunit of ricin [27] is a galactose-specific lectin that binds and is toxic to virtually all cells; thus immunotoxins containing whole ricin can be used only in the presence of an excess of the specific sugar (usually lactose) to avoid the high lectin-mediated toxicity [49]. This disadvantage can be overcome by the use of the A-chain of ricin, either purified or produced by recombinant DNA technology. Ricin A-chain retains ribosome-inactivating properties but is devoid of lectin activity, thus having a relatively low toxicity for whole cells [6, 21, 26]. More recently, a number of A-chain-like ribosome-inactivating proteins (RIPs) have been identified [3, 35, 37], and some of them (gelonin, pokeweed antiviral protein, and saporin 6) have already been used to prepare immunotoxins [37].

In the present study we evaluated a new conjugate made with Campath 1 and saporin 6 as a potential tool in the prevention of GVHD. The efficacy of Campath 1-saporin 6 immunotoxin in T lymphocyte killing and its toxicity to myeloid progenitor cells has been evaluated on the peripheral lymphomononuclear fraction (PBL) and bone marrow. Nonspecific toxicity has been evaluated both in vivo and on tumor-derived cell lines.

Materials and methods

Materials. Seeds of *Saponaria officinalis* L. (soapwort) were kindly supplied by the Botanical Officinal Garden, Casola Valsenio, RA, Italy.

N-Succinimidyl 3-(2-pyridyldithio)propionate (SPDP), Sephadex G25 superfine, G50 coarse, and Sephacryl S300 superfine were purchased from Pharmacia S. p. A., Cologno Monzese, MI, Italy. Iodogen was from Pierce Chemical Co., Rockford, Ill., USA, $Na^{125}I$ (sp. act. 13–17 mCi/μg of I) and L-^{14}C leucine (sp. act. 342 mCi/mmol) and methyl ^{3}H-thymidine (sp. act. 5 Ci/mmole) were from Amersham International, Amersham, Bucks., UK. Dimethylformamide (UV spectroscopy grade) was from Fluka AG, Buchs, CH; RPMI 1640 was from Biochrom, Berlin, West Germany; RPMI 1640 without leucine from Eurobio, Pa-

* Work supported by grants of Regione Emilia Romagna, delibera n. 1970, 13/5/86, by the Italian National Research Council, Roma, Finalized Project "Oncologia", contracts n. 86.00589.44 and n. 86.00603.44 and by the Pallotti's Legacy for Cancer Research

** Angelo Dinota is supported by a grant from Associazione Italiana per la Ricerca sul Cancro (AIRC), Milan, Italy.

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ris, France; Dulbecco's minimal essential medium (MEM) from Gibco, Paisley, Scotland, UK; fetal calf serum (FCS) from Sera Lab, Sussex, UK or from Biochrom, Berlin, West Germany; nonessential amino acids for cell culture, penicillin, streptomycin, and glutamine were from Flow, Irvine, Scotland, UK; Ficoll-Hypaque (Lymphoprep, density 1077 g/ml) was from Nyegaard, Oslo, Norway; ethidium bromide and methylcellulose were from Sigma, St. Louis, Mo., USA; phytohemagglutinin (PHA) from Wellcome, Beckenham, England, UK; vessels for cell culture were from Sterilin, Teddington, Middlesex, England, UK (microtiter test plates, 96-well trays, flat-bottomed), Falcon, Becton Dickinson, Oxnard, Calif. USA (25-cm^2 tissue culture flasks), NUNC, Roskilde, Denmark (24-cm^2 well trays and Petri dishes). Reagents for protein synthesis were obtained from the same sources as in previous work [38]. Campath 1, a rat monoclonal IgM, was kindly provided by Waldmann and Hale, Dept. of Pathology, Cambridge, UK.

Animals. Swiss female mice were from Nossan, Correzzano, MI, Italia; C57 black inbred mice were from Charles River, Calco, CO, Italy.

Purification of saporin 6. Saporin 6, purified from the seeds of *Saponaria officinalis* by ion exchange chromatography as described earlier [38] and corresponding to the material eluted as peak 6 from the carboxymethyl cellulose column, was further purified by gel filtration on a Sephadex G50 coarse column. The protein was dialyzed extensively against water, freeze dried, and stored at −20° C. Saporin 6 was labeled with ^{125}I using the Iodogen reagent according to the manufacturer's instructions.

Preparation of the immunotoxin

Saporin 6 was linked to the antibody by an artificial disulfide bond as described elsewhere [43], with some modifications. To 25 mg of antibody, dissolved in 2.2 ml of 0.9% NaCl containing 50 m*M* sodium borate buffer, pH 9.0, 30 µl of a 1.45 mg/ml solution of SPDP in dimethylformamide (corresponding to a 5-fold molar excess) was added with vigorous stirring. After 30 min at 27° C the mixture was applied to a Sephadex G 25 superfine column (30 cm × 1.6 cm) which was equilibrated and eluted with 0.14 *M* NaCl containing 5 m*M* sodium phosphate buffer, pH 7.5 at 68 ml/h at 4° C. The protein-containing effluent (16 ml) was collected and dialyzed overnight at 4° C against 2 l of 0.1 *M* NaCl containing 0.1 *M* sodium acetate buffer, pH 4.5. An aliquot of the derivatized immunoglobulin was analyzed by the method of Carlsson et al. [7]. Assuming an extinction coefficient $A_{280}^{0.1\%} = 1.24$ for Campath 1, an average of 4.7 2-pyridyldithio groups were introduced per mole of antibody. The same procedure was followed with saporin 6. Prior to derivatization a trace of ^{125}I-saporin 6 was added to 27 mg of saporin dissolved in 1.5 ml of 0.14 *M* NaCl containing 5 m*M* sodium phosphate buffer, pH 7.5 and filtered through 0.45 µm disposable filters to remove any particulate undissolved material (final sp. act. of 24,000 cpm/mol). Assuming an extinction coefficient $A_{280}^{0.1\%} = 0.6$ [23] an average of 1.49 2-pyridyldithio groups per mole of saporin were introduced. The dialyzed antibody derivative was concentrated to 3.5 ml in an Amicon ultrafiltration cell (PM 10 membrane) and then incubated at 27° C for 30 min after the addition of 0.35 ml of 0.55 *M* dithiothreitol in the same buffer. Reduction of the MoAb at pH 4.5 minimizes intrachain disulfide reductions. The mixture was applied to a Sephadex G 25 column as described before. The buffer (pH 7.5) used to equilibrate and elute the column was accurately degassed and flushed with N_2 to avoid oxidation of the reduced immunoglobulin and the consequent formation of polymers. The protein-containing effluent was collected in an Amicon ultrafiltration cell containing the saporin derivative, under constant stirring. The mixture was concentrated to 4 ml, incubated overnight at 27° C and applied to a Sephacryl S 300 superfine column (95 cm × 2.6 cm) equilibrated and eluted at 4° C with 0.14 *M* NaCl, containing 5 m*M* sodium phosphate buffer, pH 7.5 at 18 ml/h. Fractions of 2.5 ml were collected and their A_{280} and radioactivity measured. The high molecular weight (>250,000 daltons) peak containing the immunoglobulin conjugated to saporin was collected. A peak with an apparent molecular weight of 180,000–210,000 daltons was discarded. A yield of 10.63 mg of Campath 1 with an average load of 3.62 mol of saporin 6 mol of antibody was calculated. The immunotoxin was filter-sterilized through a 0.45 µm membrane and stored in aliquots at 4° C. Activity was retained for more than 3 months. An irrelevant conjugate containing a mouse IgG1 MoAb recognizing mature B cells, named 62B1 [42] and saporin 6 was obtained in the same manner as described with an average load of 2.06 mol of saporin 6/mol of antibody.

Protein synthesis. Protein synthesis was measured with a rabbit reticulocyte lysate as described previously [38] with minor modifications, with the details given in the legend to Table 1 [1]. The concentration giving 50% inhibition (ID_{50}) was calculated by linear regression analysis.

The inhibition of protein synthesis was determined with (1) a human melanoma cell line, kindly provided by Dr. A. Nichini, Istituto Nazionale Tumori, Milano, Italia, (2) NB 100 neuroblastoma cells originally from Dr. J. T. Kemshed, ICRF, London, England, (3) primary cell cultures of human neuroblastoma (AF12) (A. Pession, unpublished results), (4) TG human oviduct carcinoma cells, (5) EUE human carcinoma cells, (6) JAR human chorion-carcinoma cells, (7) BHK baby hamster kidney cells long-term cultures in the Dipartimento di Patologia sperimentale, (8) and mouse peritoneal macrophages. Cells were maintained as monolayer cultures in RPMI 1640 medium supplemented with non-essential aminoacids, 100 U/ml of penicillin, 100 µg/ml of streptomycin and 10% FCS. Mouse peritoneal macrophages were obtained from inbred 6 month old male mice of C57 black strain after intraperitoneal injection of 2 ml of 1% hydrolysed starch suspension as described by Stuart et al. [41]. Cells were seeded into 2 cm^2 wells trays, 0.5 to 1 × 10^5 cells/well, and were kept at 37° C in a humidified atmosphere containing 5% CO_2. The following day (after 4 days for mouse peritoneal macrophages) the medium was replaced by 0.5 ml serum-free RPMI with different amounts of immunotoxin, immunoglobulin or free saporin. After 18 h protein synthesis was measured by incubating the cells for 2 h (2.5 h for mouse peritoneal macrophages) in 0.5 ml of serum- and leucine-free RPMI 1640 containing 0.25 µCi of L-[^{14}C]-leucine per well (0.5 µCi in the case of macrophages). The radioactivity incorporated into protein was determined as described by Sandvig and Olsnes [36].

Toxicity. The toxicity of the immunotoxin was evaluated in Swiss female mice weighing 27–32 g, fed ad libitum. The protein, dissolved in 0.9% NaCl, was injected intraperitoneally at 5 different dose levels ranging from 0.1 to 1 mg/kg of body weight with ratio between doses of 0.178, to groups of 5 animals for each dose.

Histology. Specimens of liver, pancreas, spleen, intestine, heart, lungs, kidneys from dead animals were fixed in 3.7% formalin in saline, embedded in paraffin and stained with H&E.

Stimulation-inhibition tests. The PBL fraction was obtained from heparinized venous blood of healthy volunteers by gradient separation on Ficoll-Hypaque, for 30 min at 400 *g*. PHA-stimulated cells were cultured in 96-well microtiter flat-bottomed plate, as described elsewhere [18], with appropriate dilutions of the immunotoxin. An irrelevant immunotoxin, containing saporin 6 and mouse IgG was also used at the same concentrations. After 48 h of incubation, the cells were harvested using a Skatron equipment on paper strips and the radioactivity counted.

Culture of CFU-GM from bone marrow. Samples of heparinized bone marrow, obtained from healthy volunteers, were fractionated by Ficoll-Hypaque gradient. The cells, washed twice, were counted and checked for viability with ethidium bromide. Cells (2×10^5) were resuspended in 2.5 ml of Dulbecco's MEM supplemented with 0.9% methylcellulose, 20% FCS, and 10% of supernatant obtained from a 7-day culture of human lymphomonocytes stimulated with PHA (final concentration 1 μg/ml, PHA-conditioned medium) [19] and appropriate dilutions of immunotoxin and saporin 6. Every experiment was performed in triplicate.

The cells were plated in Petri dishes (35 × 10 mm), incubated at 37° C in a water saturated atmosphere with 5% CO_2. The CFU-GM scoring was performed on days 7, 10, 14, and 20, evaluating clusters (>20, <50 cells) and colonies (>50 cells).

Results

The activity of the immunotoxin was evaluated by its ability to inhibit protein synthesis in a cell-free system of rabbit reticulocytes. The immunotoxin was reduced at 37° C for 1 h in the presence of 0.05 *M* dithiothreitol before testing; its activity was less than that of free saporin, as observed previously [44], and compared well with that of other immunotoxins prepared with the same RIP (Table 1).

Stimulation-inhibition tests

As shown in Fig. 1 the immunotoxin effectively abolished the PHA response of PBL. In 2 different experiments performed on PHA-stimulated PBL, isolated from 2 different donors, the ID_{50} was 0.08 and 0.32 n*M*. No effect on ^{3}H-thymidine incorporation was seen with the irrelevant mouse IgG1-saporin 6 conjugate, at concentrations up to 40 n*M* (saporin 6 content); again Campath 1 and saporin 6, at concentrations up to 25 and 40 n*M* respectively, did not affect ^{3}H-thymidine incorporation.

Table 1. Characteristics and activity of the immunotoxin

	Campath 1	Saporin 6	Immunotoxin
Pyridyldithio groups inserted/mol of protein (mean)	4.70	1.49	–
Molar ratio between saporin 6 and Campath 1 (average)	–	–	3.62
Specific activity in the cell-free system of rabbit reticulocyte lysate[a] (ID_{50} ng/ml)	–	0.7	5.0[b]
Yield of conjugated protein (mg)	–	–	10.63

[a] Reaction mixtures contained in a final volume of 62.5 μl: 10 m*M* Tris/HCl buffer, pH 7.4, 100 m*M* ammonium acetate, 2 m*M* magnesium acetate, 1 m*M* ATP, 0.2 m*M* GTP, 15 m*M*-phosphocreatine, 3 μg of creatione kinase, 0.05 m*M* amino acids (minus leucine), 89 nCi of L-^{14}C-leucine, and 25 μl of a rabbit reticulocyte lysate prepared as described by Allen and Schweet [1]. Incubation was at 28° C for 5 min

[b] Related to the content of saporin 6

Toxicity evaluation on myeloid stem cells (CFU-GM)

The immunotoxin showed little toxicity to myeloid precursors, CFU-GM of the bone marrow were always present and 40% rescue was obtained at the highest concentration of immunotoxin used (Table 2).

Unrelated cell lines

The toxicity to solid tumor-derived cell lines and mouse peritoneal macrophages was evaluated from the incorpor-

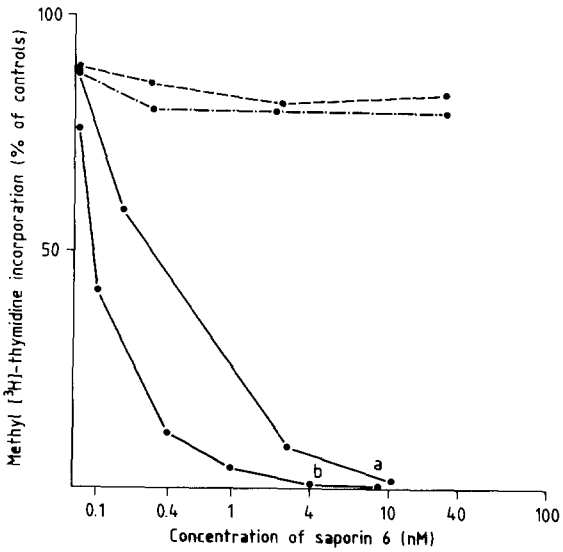

Fig. 1. Inhibition of methyl-^{3}H-thymidine uptake on phytohemagglutinin-stimulated peripheral blood lymphomononuclear fraction. Experimental conditions as described in the text. Campath 1 at the highest dose tested (25 n*M*) did not modify thymidine uptake. a) Donor 1. Campath 1-saporin 6. Control = 97,966 ±5,234. b) Donor 2. Campath 1-saporin 6. Control = 144,500 ±2,222. ------ Unrelated immunotoxin. Mean of two experiments -·-·- Saporin 6. Mean of two experiments

Table 2. Toxic effect on bone marrow cells

Immunotoxin (n*M*)	CFU-GM 14th day (clusters + colonies)	% Of controls
0	120[a]	(100)
0.58	74	62
5.8	55	46
58	48	40

[a] Mean of 2 experiments

ation of ^{14}C leucine. The inhibition of protein synthesis due to the immunotoxin was not greater than that due to the content of saporin 6 (Table 3). The only exception was a primary culture of human neuroblastoma (AF12) which showed a high sensitivity to the immunotoxin, as compared to that of the free saporin. This prompted us to look for the presence of a Campath 1-related antigen on the cell surface of this line. AF12 cells treated with Campath 1 showed intense positivity to fluorescein isothiocyanate anti rat IgM (results not shown).

Toxicity to mice

The immunotoxin showed high toxicity to mice when injected i.p. Death occurred between 48 h and 96 h after injection. A proper LD_{50} could not be calculated owing to wide variability. At 0.1 mg/kg only 1 animal survived at 14 days whereas toxicity experiments run in parallel with an IgG-saporin 6 immunotoxin gave an LD_{50} of 0.6 mg/kg, and free saporin 6 showed an ID_{50} of approximately 4 mg/kg, consistent with previous experiments done with mice of a different strain [44].

The lesions which could be observed by optical microscopy were consistent with massive hepatic necrosis; consistent signs of damage in other organs examined (spleen, gut, heart, lung, kidneys) were not found.

Discussion

Autologous or allogeneic BMT has been used in the therapy of several hematological diseases. In both instances, for successful therapy it is important to remove specific cells: malignant cells from autologous samples [9, 17], and immunocompetent cells from allogeneic samples, to avoid the onset of GVHD [12, 28, 29]. To achieve this purpose, MoAbs have been used with several modalities: (1) alone, to opsonize target cells [28]; (2) in the presence of complement, to obtain cytolysis [16, 29]; (3) conjugated with toxins, to form immunotoxins, specific to antigen-bearing cells [12, 21, 48]; and (4) linked to magnetic beads, to allow removal of the target cells [25]. No clinically significant results have been obtained with MoAbs alone [28].

The use of antibody and complement, besides requiring complement-fixing antibodies, does not achieve complete removal of antibody-binding cells, due to partial repair of the damage caused by the action of complement on the cell membrane [14]. However, this system has been used with some success to prevent GVHD, as well as for bone marrow purging before performing autologous BMT in hematological malignancies, although a longer follow-up of patients so treated is desirable.

The use of Campath 1, a monoclonal rat IgM that fixes human complement, reduced to almost zero the incidence of acute and chronic GVHD in more than 200 patients [15]. However, this kind of treatment is not free from problems: (1) the treatment is lengthy and bears the risk of cell loss; (2) the efficiency of complement-mediated lysis may vary from donor to donor, especially when human complement cannot be used; and (3) the lysis is dependent on the antigen density on the cell surface, and the cell can repair partial damage [14].

The more recently introduced immunotoxins should overcome some of the disadvantages described with the antibody-complement system. The high potency of the toxic moiety, one molecule of which is believed to kill a cell [10], may overcome the problems related to the antigen density and the possible donor complement variability.

The earliest and still most commonly used immunotoxins were made by linking the A-chain of ricin or, less frequently, of whole toxins, to MoAbs. More recently, immunotoxins constructed with different RIPs have been introduced [5, 8, 22, 24, 30–34, 45–47, 50]. These RIPs have several advantages over A-chain: (1) they are easy and safe to prepare, (2) they are not contaminated by residues of whole toxins, (3) they are stable (can be freeze-dried), and (4) they are many. Their number may be important in the case of repeated in vivo treatments to overcome the host immune response.

Our results show that an immunotoxin prepared with saporin 6 is a powerful agent in reducing the presence of normal T lymphocytes in peripheral blood cells. The analysis performed with PHA stimulation demonstrated effi-

Table 3. Effect of the immunotoxin on protein synthesis by various cell lines. When present linked or free Campath 1 was 14 n*M* and saporin 6 6.5 n*M*

Cell line	Campath 1	Saporin 6	Campath 1 + saporin 6	Immunotoxin
Protein synthesis (% of controls)				
Human melanoma	91.7	83.9	91.7	94.3
Human neuroblastoma (NB100)	91.8	77.8	86.2	86.1
Human neuroblastoma (AF12)[a]	94.1	53.2	55.3	9.8
Human carcinoma (EUE)	101.1	58.7	66.1	53.9
Human carcinoma (TG)[b]	110.1	24.8	30.9	35.9
Human chorioncarcinoma (JAR)	101.0	80.7	77.4	82.0
Baby hamster kidney cells (BHK)	96.6	81.8	not determined	91.2
Mouse peritoneal macrophages	84.8	27.9	23.3	28.2

[a] Mean of two experiments
[b] Mean of three experiments

cient killing of T lymphocytes. The toxicity toward myeloid stem cells (CFU-GM) was similar to that observed with other immunotoxins [11]. Immunotoxins made with ricin have been reported to reduce the number of CFU-GM from 50% to 70% of the control value [11]: the same inhibition was observed with the Campath 1-saporin 6 immunotoxin, although at high concentrations the toxicity increased. It should be noted that Campath 1-saporin 6 immunotoxin was present in the culture media for more than 10 days whereas the data reported for ricin-containing immunotoxins were usually obtained with shorter incubation times (4–20 h) [11].

The immunotoxin described in this paper is one of the few prepared with IgM MoAbs. The use of an IgM could offer the advantage of a higher load of RIP (5 mol/mol of IgM, as compared to 1–2 mol/mol of IgG) without alteration of the antigen-binding capability of the antibody. This allows the delivery of a greater number of active molcules per mole of cell-bound antibody. Entry into the cytoplasm does not seem to be hindered by the larger mass of the complex, as shown by the inhibition of protein synthesis of target cells.

On the other hand, the immunotoxin was highly toxic when administered to mice. The increased toxicity as compared to that of free saporin is probably due to a slower clearance from the bloodstream of saporin once linked in a high molecular weight complex [33, 39, 44]. It has already been shown that this is probably the case with IgG-saporin conjugates [44]. This high toxicity is a severe limitation to the in vivo use of IgM-containing immunotoxins, although in humans IgM has a shorter half-life than IgG, which is not governed by size. However, they could be suitable for the ex vivo purging of bone marrow. In fact, for a bone marrow buffy coat with a volume of 200–300 ml, 60–90 μg of Campath 1-saporin 6 (expressed as saporin 6 content) is needed to obtain a concentration of 10 n*M*, which is the optimal dose active in killing Campath 1-positive cells (Fig. 1). This amount of conjugated saporin 6 (1–2 μg/kg of body weight) can be reduced 10-fold by centrifuging the buffy coat, after incubation, allowing a significant reduction in the amount of immunotoxin administered to the patient.

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Received October 19, 1987/Accepted January 12, 1988