Evidence for Absence of Toxicity of T101 Immunotoxin on Human
Hematopoietic Progenitor Cells Prior to Bone Marrow
Transplantation


ABSTRACT

T101-ricin A-chain immunotoxin is a hybrid molecule made up of the T101 monoclonal antibody bound to the A-chain of ricin. It specifically destroys cells expressing the cell surface T65 antigen. We have designed a preclinical study to evaluate its possible use for the in vitro treatment of T-cell hematological cancers prior to autologous bone marrow transplantation. The data presented here show that conditions previously defined to produce high tumor cell killing, i.e., a 20-hr incubation at 37 °C in the presence of T101-ricin A-chain immunotoxin up to 10^{-7} M in a 10 mM ammonium chloride solution, do not affect the in vitro proliferative capacity of human hematopoietic stem cells studied by means of semisolid medium cultures (granulocyte-macrophage progenitors, burst-forming units-erythrocyte) and continuous liquid cultures (pro-granulocyte-macrophage progenitors). Therefore, autologous bone marrow transplantation with T101-ricin A-chain immunotoxin-treated graft should be feasible.

INTRODUCTION

Monoclonal antibodies have been used recently in an attempt to purge leukemic bone marrows, collected in remission, of their few residual tumor cells, (21, 27). Indeed, many of the obstacles limiting their use in vivo, such as the presence of circulating antigens, antigenic modulation, reactivity with normal cells, or immune response to murine antibody, may be circumvented through the carefully controlled manipulation of the reagents in vitro. However, these techniques require important bone marrow processing and the use of heterologous complement, the non-specific toxicity of which may result in the loss of normal hematopoietic stem cells.

Monoclonal antibodies may be now used as carriers of various cytotoxic agents. Specific procedures to conjugate to antibodies chemotherapeutic agents (1, 10), lipid vesicles loaded with either drugs or toxins (17), or plant toxins (28) such as ricin A-chain (2, 11, 15, 16, 20, 25) are presently available.

We report here the results of a preclinical study designed to evaluate the possible toxicity on human hematopoietic stem cells of T101-IAT,3 consisting of the A-chain subunit of ricin linked to the T101 monoclonal antibody, in view of its possible application to the in vitro treatment of bone marrow in patients with T-cell cancers prior to autologous bone marrow transplantation.

MATERIALS AND METHODS

Immunotoxin T101. T101-IAT (Centre de Recherches Clin-Midy-Sanofi, Montpellier, France) is an hybrid molecule made up of the A-chain subunit of ricin linked to the T101 monoclonal antibody described by Royston. T101-IAT contains a mean of 2.5 A-chains per antibody molecule (4). T101, an IgG2a globulin, reacts with the T65 antigen present on the surface of all normal T-cells, most thymocytes, T-cells of acute lymphocytic leukemias, and leukemic T-cell lines (22) and also on B-cells of chronic lymphocytic or prolymphocytic leukemias (23). The T101 binds the T65 differentiation antigen which allows the A-chain of ricin to cross the membrane. Then the A-chain penetrates the cytoplasm, inactivates ribosomes, and blocks protein synthesis irreversibly, inducing cell death (16).

Patient Population and Bone Marrow Collection. Bone marrow samples were harvested by aspiration from iliac spines in patients either with T-cell cancers in complete remission or with solid tumors without bone marrow involvement at the time of collection in the surgery room for autologous bone marrow transplantation.

Bone Marrow Treatment with T101-IAT. Volumes of 50 or 100 ml of freshly aspirated marrow were used for the following in vitro studies. The total bone marrow (i.e., without any cell separation) was incubated in a 150-ml bag (GAMBRIO DF 1000, Heschingen, West Germany) at 37 °C in a 10 mM ammonium chloride solution with increasing dosages of T101-IAT, expressed as final A-chain concentration in molarity, with gentle continuous shaking. Marrow samples were drawn at various intervals (1 to 20 hr) for hematopoietic stem cell assay.

Bone Marrow Freezing Technique. After the incubation period, bone marrow was mixed with an equal volume of freezing medium consisting of TC 199 with glutamine and salts of Earle's medium (Gibco/Biocult, Glasgow, Scotland), 20% dimethyl sulfoxide (Assistance Publique, Paris, France), and 10% matched human serum. The final concentrations were 10% for dimethyl sulfoxide and 5% for serum. This mixture was then frozen according to previously described techniques (6). Bone marrow was frozen in the vapor phase of liquid nitrogen in a Niccol biological freezing system (Air Liquide, Grenoble, France). The freezing rate was 1 °C/min until the release of the heat of fusion at which time the temperature inside the freezing chamber was sharply diminished. A particular attention was devoted to the third slope in order to keep a cooling rate slower than −5 °C/min after the transition phase (7, 13). The marrow was then stored in the gas phase of liquid nitrogen at −196 °C.

After thawing in a waterbath at 37 °C, specimens were drawn for in vitro assays of the cryopreserved stem cells.

Culture of CFUc. CFUc assay was performed in semisolid agar by a modification of the technique of Pike and Robinson (19). The basic medium was McCoy's Medium 5A without serum (Gibco/Biocult) supplemented with 30% decomplemented FCS (Gibco/Biocult). CSA was sup-

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3 The abbreviations used are: T101-IAT, T101-ricin A-chain immunotoxin; FCS, fetal calf serum; BFUe, granulocyte-macrophage progenitors; BFUs, burst-forming unit-erythroblast; CSA, colony-stimulating activity.

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plied by 10% human placental conditioned medium (kindly provided by Dr. R. Berthier, CEN, Grenoble, France), produced according to the method of Schlunk and Schleyer (24). The same batches of FCS and human placental conditioned medium were used for all of the studies.

The mononuclear bone marrow cells were isolated over a Ficoll-sodium metrizoate gradient (d = 1.077; Lymphoprep; Nyegaard, Oslo, Norway). They were cultured in 35- x 10-mm Petri dishes (Corning), using 2 x 10^6 bone marrow cells/ml of medium containing equal volumes of 0.6% agar and McCoy’s Medium 5A 2x (Gibco/Biocult) to achieve a final serum concentration of 15%. Three Petri dishes were plated for each assay.

The cultures were incubated for 10 days at 37°C in a humidified 5% CO2 atmosphere. The colonies were examined on the Petri dishes by means of an inverted microscope, and counts were made of colonies containing more than 50 cells. The results were expressed in terms of the number of colonies per ml of marrow.

Culture of BFUe. BFUe assay was performed in methylcellulose by a modification of the technique of Iscover and Sieber (14). Mononuclear cells (2 x 10^5) isolated above were cultured in triplicate in 0.9% bovine serum albumin (Sigma), 20% NCTC (Eurobio, Paris, France) and 1 unit pork erythropoietin (CNTS, Paris, France). Cultures were incubated for 14 days at 37°C in a humidified 5% CO2 atmosphere. Colonies were examined on the Petri dishes through an inverted microscope, and counts were made of colonies containing more than 40 cells. The erythroid nature of the cells in colonies was confirmed by examination of picked colonies stained with May-Grünwald-Giemsa. The results were expressed in terms of the number of colonies per ml of marrow.

Continuous Liquid Cultures. Long-term liquid cultures were established using a modification (8) of the technique of Dexter et al. (5). Bone marrowuffy coat cells (10^6) from normal donors were suspended in 1 ml of α medium (Gibco/Biocult) supplemented with 20% FCS. Five ml of this suspension were put into Falcon 25-sq cm tissue culture flasks, and the suspension was incubated at 37°C in a 5% CO2 atmosphere. Cultures were maintained by weekly demipopulation with addition of fresh medium. Under these conditions, an uniform adherent layer spread within 3 to 4 weeks. Then, this primary culture was depopulated of all suspension cells and irradiated at 30 Gy. A new allologous inoculum of bone marrow cells was added onto the adherent layer. Cultures were maintained by replacing all the medium weekly, without demidepopulation. At the same time, suspension cells were assayed for CFUc as described above.

RESULTS

The sensitivity of normal progenitors was evaluated by their ability to proliferate. Results were expressed in terms of the percentage of stem cells recovered per ml of manipulated marrow, by comparison with nonmanipulated marrow. The results shown represent the mean ± S.D. of 3 to 9 determinations for each point. Sequential studies with incubation of the marrow for periods up to 20 hr were done for each individual patient. In these experiments, the mean number of colonies detected in controls were, respectively, for CFUc and BFUe: 124 ± 38 and 149 ± 52/2 x 10^6 mononuclear cells or 1420 ± 340 and 1603 ± 2583/10^3/ml bone marrow. These values represent what is usually expected for normal marrow in our experience.

Conditions previously shown to produce high tumor cell killing were tested (5). Table 1 shows the recovery of CFUc and BFUe after the incubation of the marrow at 37°C for a period up to 20 hr in the presence of 10 mM NH4Cl. As indicated, NH4Cl does not alter the viability of CFUc or BFUe. Moreover, a slight increase in CFUc and BFUe is observed. To test the influence of these experimental conditions further on the sensitivity of progenitor cells to cryopreservation, we then compared the recovery following freezing and thawing of incubated marrow to the recovery of cryopreserved nonincubated marrow. CFUc recovery of marrow incubated for 20 hr with 10 mM NH4Cl dropped to 33 ± 9%. BFUe were somewhat more sensitive with a recovery of 32 ± 16%.

Marrows were then incubated in those same conditions with increasing doses of T101-IAT, ranging from 10^-9 to 10^-6 M. Results are shown on Table 2. No effect of T101-IAT on progenitor cells proliferation was observed after a 4-hr incubation up to 10^-6 M. After a 20-hr incubation, toxicity was seen at the 10^-6 M level. BFUe progenitors were more sensitive to T101-IAT than was CFUc. After cryopreservation, CFUc and BFUe recovery again was reduced but in a way similar to that observed previously for marrows either nontreated or NH4Cl incubated. The ability of T101-IAT treated marrow to maintain myelopoiesis in continuous marrow culture was tested. Frozen bags of marrows incubated with 10^-4 M T101-IAT for 20 hr at 37°C in a 10 mM NH4Cl solution were maintained over the primary irradiated allogenic adherent layer. No impairment in CFUc generation (Table 3) was seen in the T101-IAT-treated group. Moreover, a small but significant improvement in the extent of CFUc production was evident at Weeks 1, 2, and 3, as compared to

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erythrocytes and granulocytes do not bind T101-IAT nonspecifically (26), demonstrating the absence of T65 toxicity up to $10^{-7}$ M. These results are consistent with those of Gering into DMA synthesis. T101-IAT itself has no significant toxicity up to 20 hr in the presence of 10 mM NH4CI does not affect the in vitro proliferation of committed progenitor cells. Moreover, the slight decrease of CFUc and BFUe within 1 hr, immediately followed by a secondary increase, may be attributed to a triggering into DNA synthesis. T101-IAT itself has no significant toxicity up to $10^{-7}$ M. These results are consistent with those of Teatle and Royston (26), demonstrating the absence of T65 antigen on normal CFUc and BFUe. They also confirm the results of Casellas et al. (4) showing the lack of nonspecific toxicity of T101-IAT on the Daudi cell line (T65 negative) up to $10^{-7}$ M. The slight sensitivity of BFUe may be related to the absence of T-cells, the presence of which could be required for their in vitro proliferation in semisolid media culture (18).

Our results have shown that the incubation period at 37°C up to 20 hr in the presence of 10 mM NH4CI does not affect the in vitro proliferation of committed progenitor cells. Moreover, the slight decrease of CFUc and BFUe within 1 hr, immediately followed by a secondary increase, may be attributed to a triggering into DNA synthesis. T101-IAT itself has no significant toxicity up to $10^{-7}$ M. These results are consistent with those of Teatle and Royston (26), demonstrating the absence of T65 antigen on normal CFUc and BFUe. They also confirm the results of Casellas et al. (4) showing the lack of nonspecific toxicity of T101-IAT on the Daudi cell line (T65 negative) up to $10^{-7}$ M. The slight sensitivity of BFUe may be related to the absence of T-cells, the presence of which could be required for their in vitro proliferation in semisolid media culture (18).

DISCUSSION

This study was designed to evaluate the potential toxicity of T101-IAT on human hematopoietic stem cells under conditions previously defined to produce high tumor cell killing. The efficacy of T101-IAT has been measured previously by inhibition of protein synthesis through the uptake of $[^{14}C]$leucine into CEM cells (human T-leukemic cell line expressing T65) (3) and by the evaluation of the surviving cells determined by cloning assay in agar (4). The optimal conditions for a reliable and highly efficient elimination of leukemic cells from autologous graft were established as follows (3, 4). A 99.90% elimination of leukemic cells among a 10-fold excess of bone marrow cells was obtained after a 4-hr incubation at 37°C in a 10 mM NH4CI solution with doses of $10^{-8}$ m ITT101. However, after a 20-hr incubation, elimination rose to 99.99%. We therefore selected this duration for cleansing the marrow. NH4CI (10 mM) has been shown to have a highly potentiating effect. It acts on the immunotoxin internalization process by increasing intralysosomal pH which blocks the lysosomal pathway of protein degradation (3). Therefore, the activation can be attributed to an inhibition of protein degradation.

Unfractionated bone marrows were used rather than mononuclear cell suspension for practical purposes, mainly to prevent a loss of stem cells which might result from additional steps such as spinnings and washings and because it has been shown that erythrocytes and granulocytes do not bind T101-IAT nonspecifically (3).

Results have shown that the incubation period at 37°C up to 20 hr in the presence of 10 mM NH4CI does not affect the in vitro proliferation of committed progenitor cells. Moreover, the slight decrease of CFUc and BFUe within 1 hr, immediately followed by a secondary increase, may be attributed to a triggering into DNA synthesis. T101-IAT itself has no significant toxicity up to $10^{-7}$ M. These results are consistent with those of Teatle and Royston (26), demonstrating the absence of T65 antigen on normal CFUc and BFUe. They also confirm the results of Casellas et al. (4) showing the lack of nonspecific toxicity of T101-IAT on the Daudi cell line (T65 negative) up to $10^{-7}$ M. The slight sensitivity of BFUe may be related to the absence of T-cells, the presence of which could be required for their in vitro proliferation in semisolid media culture (18).

Since committed stem cells are an indirect reflection of the self-renewing capacity of the totipotent stem cells, we have studied the behavior of T101-IAT-treated and frozen marrows in continuous liquid cultures. Results demonstrate the lack of toxicity on pre-CFUc progenitors of T101-IAT at doses used in clinics ($10^{-5}$). Indeed, a greater number of CFUc colonies was detected at Weeks 1, 2, and 3 in cultures of treated marrows as compared to controls. It has been demonstrated (9) that depletion of some T-lymphocyte subsets may significantly alter the balance between differentiation and proliferation of CFUc possibly because of a modification in CSA level. Therefore, one may postulate that initial destruction of relatively mature T-cells by T101-IAT, leading to CSA level depression, slows differentiation of CFUc which hence are maintained longer in culture. Subsequently, T-cells regenerate from pluripotential stem cells, as shown from pluripotential stem cells in semisolid medium culture analysis (4), which explains at Week 4 the secondary drop in CFUc.

Therefore, these experimental conditions for T101-IAT allow the in vitro treatment of bone marrow potentially contaminated with residual leukemic cells prior to autologous bone marrow transplantation. A trial using T101-IAT in vitro has been initiated in 4 patients with T-cell cancers. All patients have engrafted within normal limits, in spite of sensitization of progenitors to cryoinjury, which may be related to the incubation procedure itself and not to the action of T101-IAT.

The use of T101 immunotoxin A-chain may also be extended to the setting of allogenic bone marrow transplantation, through the elimination of T-cells for prevention of graft-versus-host disease (12).

Monoclonal antibodies as carriers for toxins are a promising tool for a selective removal of leukemic cells which may significantly increase the clinical effectiveness of antibodies in vitro, without any nonspecific toxicity.

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REFERENCES


Table 3

<table>
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<th>CFUc/ml culture</th>
<th>T101-IAT treated frozen marrows</th>
<th>Nontreated frozen marrows</th>
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*Mean ± S.D. of 3 separate experiments.*

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