Optimal Elimination of Leukemic T Cells From Human Bone Marrow With T101-Ricin A-Chain Immunotoxin

By Pierre Casellas, Xavier Canat, Axel A. Fauser, Olivier Gros, Guy Laurent, Philippe Poncelet, and Franz K. Jansen

Progress has been made in rendering monoclonal antibodies highly cytotoxic by coupling them to the A-chain subunit of ricin or diphtheria toxin.1,2 Such conjugates, called immunotoxins (ITs), which possess the dual properties of specificity and cytotoxicity toward target cells, were less active than the intact toxin.3,4

Recently, it was described that lysosomotropic amines or carboxylic ionophores strongly increased the specific toxicity of ITs, resulting in an efficiency that was even higher than that of ricin.5,6

These findings show that ITs could be of great value for the selective elimination of leukemic cells in vitro. Thus, they should be clinically useful for purging of bone marrow in autologous bone marrow transplants.

One particularly promising reagent is the immunotoxin prepared with the monoclonal antibody T101, which reacts with the T cell antigen, T1, a 65-kD glycoprotein that is expressed on mature T lymphocytes, most T cell-derived hematologic malignancies, and some chronic B lymphocytic leukemias. It is not expressed on normal myeloid and erythroid progenitor cells.7

Before any clinical application of IT can be envisaged, it is extremely important to determine the potency of such reagents. Sensitive in vitro analysis of the ability of IT to kill specific target cells is critical, considering that complete elimination of leukemic cells from the grafts may be essential for successful bone marrow transplantation.

In this article we use a sensitive and quantitative clonogenic assay for the measurement of the number of residual target cells. Variables such as incubation time, temperature, IT concentration, cellular concentration, and presence of the human bone marrow elements were examined in order to establish conditions for optimal and specific killing effect. The cytoreduction of target cells thus obtained exceeded 6 logs with the T101 IT; therefore, a clinical study of purging presence of 10 mmol/L NH4Cl. This treatment led to the reduction of more than six orders of magnitude of T101-positive clonogenic leukemic cells, with no harm to T101-negative cells. Moreover, we observed no toxicity of IT to human hematopoietic stem cells (CFU-GEMMT) derived from bone marrow of healthy volunteers. Thus, pretreatment of bone marrow samples with IT plus NH4Cl offers a safe, simple, reliable, and highly efficient means to eliminate undesirable leukemic T cells from the graft.

MATERIALS AND METHODS

Cell Lines

All cell lines used were kindly provided by Dr Carrel (Ludwig Institute, Lausanne, Switzerland): CEM, a human T leukemia cell line carrying the T cell antigen, T1, established by Foley et al,8 a Daudi cell line established by Klein et al,9 and a Raji cell line established by Pulferaft et al10 (two human lymphoblastoid B cell lines that originated from Burkitt’s lymphomas). Cells were cultured at 37°C in a 5% CO2 atmosphere in a basal culture medium consisting of RPMI 1640 medium (Merieux, Lyon, France) supplemented with 10% heat-inactivated fetal calf serum (Flow Labs, McLean, Va) and antibiotics (streptomycin, 100 μg/mL, and penicillin, 100 U/mL).

Human Bone Marrow

Bone marrow was obtained from healthy volunteer donors. Marrow was aspirated from the sternum, which was punctured with a syringe containing heparin. The marrow contained approximately 2 x 107 nucleated cells/mL and was used without gradient cell separation. In addition, bone marrow was aspirated into heparinized syringes from the iliac crest of consenting healthy volunteers.

Immunotoxins

Two ITs were prepared: an anti-human T cell IT, using the mouse monoclonal IgG2a antibody, T101,11 purchased from Hybritech (San Diego), and an anti-carcinoembryonic antigen IT, using the mouse monoclonal IgG1 antibody, 3517,12 generously donated by Dr J.P. Mach (Ludwig Institute). These two ITs will be referred to as T101 IT and anti-CEA IT, respectively. Monoclonal antibodies were purified from mouse ascitic fluid by affinity chromatography on a column of Staphylococcus aureus Protein A coupled to Sepharose.

From the Department of Immunology, Centre de Recherches Clin Midy (groupe Sanoﬁ), Montpellier, France, and the Medizinische Universitas-Klinik, Freiburg, FRG.

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Address reprint requests to Dr Pierre Casellas, Centre de Recherches Clin Midy (Groupe Sanoﬁ), Rue du Professeur J. Blayac, 34082 Montpellier, France.

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Ricin A-chain was conjugated to antibodies according to the method previously described.\textsuperscript{13} Final products were routinely assayed by sodium dodecyl sulfate (SDS) gel electrophoresis for molecular weight determination. Enzymatic A-chain activity was measured by the capacity of the toxin subunit to inhibit protein synthesis in a cell-free system.\textsuperscript{14} Antibody binding was assessed by fluorescence-activated cell sorter (FACS IV, Becton Dickinson, Mountain View, Calif) analysis on the target cells.\textsuperscript{4} Cytotoxicity was determined by inhibition of cell protein synthesis according to the methods previously described.\textsuperscript{6,13} As judged by these methods, all A-chain and antibody activities were preserved after conjugation, and ITs were stable for over one year at 4°C in phosphate-buffered saline, pH 7.4. The average number of A-chain molecules per antibody was in each case in the range of 1.5 to 2.0. IT concentration was expressed as an A-chain equivalent molarity.

**Immunotoxin Treatment of Clonogenic Leukemic Cells**

One million cells taken in the exponential phase of growth were treated with IT at the final concentration indicated in the figures, in the presence of 10 mmol/L NH₄Cl in a total volume of 1 mL. Cells were incubated at 37°C in 5% CO₂ atmosphere with gentle agitation (250 rpm on a Gyroraryy G-2, New Brunswick Scientific, Edison, NJ) for a given time, as indicated in each experiment. As controls, cells were identically treated, except for the omission of IT.

When leukemic cells were treated in the presence of bone marrow, they were centrifuged and then resuspended in crude marrow and exposed to IT as mentioned previously. At the end of incubation, IT-treated cells or controls were thoroughly washed (three times) to remove IT, then reasuspended in the cloning medium (see later). Following washing, various cell suspensions were prepared, ranging from 10⁵ to 1 cell/0.2 mL for the cloning assay. When the assay was performed in the presence of bone marrow, red blood cells were first lysed with a salt solution (155 mmol/L NH₄Cl, 8 mmol/L KHCO₃, 1 mmol/L EDTA); this treatment did not affect the cloning efficiency of clonogenic leukemic cells.

**Cloning Assay for Measurement of Cytotoxicity**

The cloning assay was a modification of the double agar layer (agarose type VII, Sigma, St Louis) cloning method of N.K. Ayres.\textsuperscript{14} The cloning medium consisted of RPMI 1640, 1 mmol/L alpha-ketoglutarate (Sigma), 1 mmol/L sodium oxalacetate (Sigma), 5% heat-inactivated fetal calf serum, and 10% heat-inactivated horse serum (Flow). The agar concentration that was optimal for both cloning frequency and for the size of the clones in order to be easily scorable with an automatic colony counter was determined. The bottom layer consisted of 2.5 mL of 0.3% agarose solution in cloning medium in 60-mm tissue culture dishes (Corning, Corning, NY). The second layer was prepared by mixing 2 mL of 0.3% agar solution with 0.2 mL cell suspension containing various cell numbers ranging from 1 to 10⁶ cells and poured onto the bottom layer. Tissue culture dishes were incubated at 37°C for 15 to 20 days, after which colonies were scored using an automatic colony counter (Artrek 980 System, Dynatech, Farmingdale, NY). Results were expressed as an absolute number of surviving cells extrapolated from the cloning frequency.

Absolute cell survival was determined by the equation:

\[
C \times d \leq F^{-1},
\]

where \(C\) is the number of clones per Petri dish, \(d\) is the dilution tested, and \(F\) is the cloning frequency, established as the number of colonies scored divided by the number of control cells plated. Each point corresponded to the mean of triplicate dishes.

**Surface Marker Analysis of Leukemic Cells**

Clones generated from surviving cells were isolated using a Pasteur pipette, dispensed into microtiter plates, and grown in culture medium. After one to two weeks, cells were analyzed for their expression of the T1 antigen. The number of T1 molecules per cell was assayed by comparison with other CEM subclones whose T10 binding levels (1,000, 5,000, 20,000, and 44,000, respectively, molecules per cell) had previously been determined by saturation experiments using radiolabeled T101. These cell lines, whose mean fluorescence intensity had been shown to be proportional to the number of T101 molecules bound per cell, were analyzed together with the test samples and served to build a standard curve for quantification (Poncelet et al, manuscript in preparation).

**Sensitivity of Cells to the Immunotoxin**

The sensitivity of the cells to the IT was determined by the inhibition of protein synthesis according to the method described.\textsuperscript{4} The concentration of IT that produced 50% inhibition (denoted IC₅₀) was used for comparison.

**Preparation of Leukocyte Conditioned Medium**

Conditioned medium was prepared from peripheral leukocytes from a patient with hemochromatosis.\textsuperscript{15} Briefly, 10⁶ mononuclear cells with a density of less than 1.007 g/mL were incubated in 1 mL of a medium containing 1% human serum albumin (HSA) (Sigma) and 1% phytohemagglutinin (PHA) (Burroughs Wellcome, Research Triangle Park, NC) in Iscove's modified Dulbecco's medium (JJDM) GIBCO Laboratories, Grand Island, NY). This material, called PHA-LCM, was harvested after four days of incubation.

**Immunotoxin Treatment of Bone Marrow Cells**

Aliquots of 10⁶ mononuclear bone marrow cells/mL obtained by centrifugation of Ficoll-Hypaque (Pharmacia Fine Chemicals, Upssala, Sweden) were incubated over periods of eight and 24 hours, respectively, at 28°C or 37°C in a humidified atmosphere at 5% CO₂ under gentle agitation. The cells were treated with T101 IT, T101 IT plus 10 mmol/L NH₄Cl, ricin A-chain, and ricin A-chain plus 10 mmol/L NH₄Cl, in concentrations ranging from 10⁻⁷ to 10⁻⁴ mol/L. In addition, bone marrow cells were incubated in the presence of the T101 monoclonal antibody in concentrations of 4.00, 0.40, 0.04 μg/mL.

As controls, aliquots were identically treated without the addition of IT or A-chain. At the end of the incubation period, bone marrow cells were washed in IMDM and resuspended to the original volume.

** Colony Assay for Hematopoietic Progenitors (CFU-GEMMT, BFU-E, and CFU-C)**

Mixed hematopoietic colonies (CFU-GEMMT),\textsuperscript{18,17} erythroid bursts (BFU-E),\textsuperscript{14} and granulocyte colonies (CFU-C)\textsuperscript{19} were grown as previously described. Aliquots of 200 μL of treated mononuclear cells were admixed with 30% fresh human plasma (FHP), 5% PHA-LCM, 2 × 10⁻⁵ mmol/L mercaptoethanol, IMDM, and 0.9% methylcellulose. In order to obtain a significant number of multilineage colonies, cultures were plated in quadruplicates. The incubation was performed at 37°C in a humidified atmosphere at 5% CO₂. After four
days of incubation, 1 unit of erythropoietin (Connaught, Toronto, step III) was added to each culture.

Multilineage hematopoietic colonies were identified at day 14 by their morphological appearance, containing cells with the red color typical for hemoglobin, admixed with colorless and translucent cells of various sizes. For further analysis, mixed hematopoietic colonies were removed from the cultures by means of a micropipette.

T Cell Markers on Mixed Hematopoietic Colonies

Individual colonies were aspirated with a micropipette and washed in Eppendorf micro-test tubes. Each pellet was resuspended after centrifugation (five minutes, 300 g). Aliquots were transferred onto poly-1-lysine-coated glass slides for staining of surface antigens achieved with the T leukemic cell line, CEM, seeded in 300 μL. After centrifugation (five minutes, 10 g), pellets were transferred into Eppendorf micro-test tubes. Each pellet was resuspended by a double agar layer under conditions described in Materials and Methods. The monoclonal antibody T101 was used for surface staining. Controls were performed with anti-β2-microglobulin and the different sandwich antisera, rabbit anti-mouse (RAM) and swine anti-rabbit (SAR).

RESULTS

Cloning Assays for Measurement of Cell Killing

Cell killing induced by IT was usually assessed by measurement of protein synthesis inhibition. This assay was not sensitive enough to measure the extent of cell killing (several log), which is expected with different sandwich antisera, rabbit anti-mouse (RAM) and swine anti-rabbit (SAR). To generate dead cells, CEM cells were treated with IT plus 10 mmol/L NH₄Cl, at which all target antigens were occupied (see later). In Fig 2, the number of surviving cells decreased as a function of the length of IT treatment (14% after 24 hours). When cells were treated with IT plus 10 mmol/L NH₄Cl, more dramatic results were obtained, as 99.95% of cytoreduction could be achieved at four hours and even 99.99% at eight or 24 hours. In contrast, treatment of the cells with ammonium chloride alone was not toxic. This demonstrated the potentiating effect previously described.

Effect of Incubation Time, Temperature, and Addition of Ammonium Chloride on T101 IT Cell Killing

CEM cells were treated for varying time intervals with T101 IT at a dose of 10⁻⁴ mol/L, at which all target antigens were occupied (see later). In Fig 2, the number of surviving cells decreased as a function of the length of IT treatment (14% after 24 hours). When cells were treated with IT plus 10 mmol/L NH₄Cl, more dramatic results were obtained, as 99.95% of cytoreduction could be achieved at four hours and even 99.99% at eight or 24 hours. In contrast, treatment of the cells with ammonium chloride alone was not toxic. This demonstrated the potentiating effect previously described.

![Fig 2. Effect of incubation time and presence of ammonium chloride on cytotoxicity. Cells (10⁶/mL) were incubated at 37 °C with T101 IT (10⁻⁴ mol/L) in the presence (●) or absence (□) of 10 mmol/L NH₄Cl or with 10 mmol/L NH₄Cl alone (●). At each time interval indicated on the abscissa, cells were washed and plated in triplicate at different cell concentrations. Standard curves of cloning frequency were run with untreated cells for each time and used for calculations of cell survival as described in Materials and Methods.](http://www.bloodjournal.org)
Temperature of treatment also affected the susceptibility of the cells to the IT effect. As shown in Table 1, maximum effect was obtained at 37 °C. When the treatment was carried out at 25 °C or 8 °C for eight hours, the frequency of cell survival increased 10²- and 10³-fold, respectively, as compared with that at 37 °C. The inhibitory effect of the temperature could not be compensated for by a prolonged incubation period. Conditions that gave optimal cell elimination, ie, 37 °C for eight or 24 hours in the presence of 10 mmol/L NH₄Cl, were chosen for subsequent experiments.

Table 1. Effect of the Temperature of Treatment on IT Cell Killing

<table>
<thead>
<tr>
<th>Duration of Treatment</th>
<th>Temperature of Treatment</th>
<th>Cell Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 h</td>
<td>8 °C</td>
<td>14.00</td>
</tr>
<tr>
<td>8 h</td>
<td>25 °C</td>
<td>0.90</td>
</tr>
<tr>
<td>8 h</td>
<td>37 °C</td>
<td>0.01</td>
</tr>
<tr>
<td>24 h</td>
<td>8 °C</td>
<td>12.00</td>
</tr>
<tr>
<td>24 h</td>
<td>25 °C</td>
<td>0.10</td>
</tr>
<tr>
<td>24 h</td>
<td>37 °C</td>
<td>0.01</td>
</tr>
</tbody>
</table>

CEM cells were treated at 8 °C, 25 °C, or 37 °C with T1O1 IT (10⁻⁸ mol/L) for eight or 24 hours in the presence of 10 mmol/L NH₄Cl.

Effect of T1O1 IT Concentration on CEM Cells and on CEM Cells Mixed With Human Bone Marrow

CEM cells alone or added to nonseparated human bone marrow containing about a tenfold excess of nucleated cells were treated with T1O1 IT over a wide range of concentrations. Cell survival was then measured by cloning assay. Results are shown in Fig 3. Killing of CEM cells was as effective whether or not human bone marrow was present. This demonstrated that the presence of normal hematopoietic elements did not inhibit IT efficacy.

The number of CEM cells was sharply reduced by the treatment with T1O1 IT at each concentration tested. Conversely, it was not affected by the treatment with the unrelated anti-CEA IT. Reduction in the number of viable cells recovered was observed only at the highest anti-CEA IT concentration. This suggested that up to 10⁻⁸ mol/L the effect of T1O1 IT was specific.

A direct proof of this conclusion was given by the fact that pretreatment of cells with an excess of T101 unconjugated antibody could completely block T101 IT cell killing. On the other hand, antibody alone at saturating concentration was not toxic (Table 2). This demonstrates that specificity and cytotoxicity of IT are mediated by the antibody and the A-chain moieties, respectively.

The addition of 0.1 mol/L lactose, which blocks the galactose binding site of the ricin B-chain, did not diminish the nonspecific cytotoxicity at all concentrations tested; this excluded the possibility that nonspecific cytotoxicity could be due to any B-chain contamination (Table 3).

It should be noted that the presence of 10 mmol/L NH₄Cl induced only a slight stimulation of the cytotoxic activity of the unrelated anti-CEA IT (a factor of 3 at the highest dose tested) (Table 3). This effect was
Table 3. Effect of Lactose and Ammonium Chloride on Nonspecific Cytotoxicity

<table>
<thead>
<tr>
<th>Concentration of Immunotoxin (mol/L)</th>
<th>Cell Survival (%)***</th>
<th>+ NH₄Cl</th>
<th>+ NH₄Cl + Lactose</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>10⁻⁶</td>
<td>-</td>
<td>98</td>
<td>99</td>
</tr>
<tr>
<td>10⁻⁷</td>
<td>-</td>
<td>95</td>
<td>60</td>
</tr>
<tr>
<td>10⁻⁸</td>
<td>-</td>
<td>90</td>
<td>25</td>
</tr>
</tbody>
</table>

10⁶ CEM cells were exposed for 24 hours at 37 °C to anti-CEA IT at various doses, with or without mmol/L NH₄Cl, or with 10 mmol/L NH₄Cl plus 0.1 mol/L lactose. Cells were washed and plated in triplicate at different cell concentrations.

*The percentage of cell survival was calculated from untreated controls. Values represent the mean of triplicate wells for each treatment.

Table 4. Effect of TiOl IT on Clonogenic T1-Negative Cells Mixed With Human Bone Marrow

<table>
<thead>
<tr>
<th>Concentration of Immunotoxin (mol/L)</th>
<th>Cell Survival (%)***</th>
<th>Daudi Cells</th>
<th>Raji Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁻⁶</td>
<td>-</td>
<td>130.0</td>
<td>110</td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>-</td>
<td>105.0</td>
<td>100</td>
</tr>
<tr>
<td>10⁻⁴</td>
<td>-</td>
<td>25.0</td>
<td>70</td>
</tr>
<tr>
<td>10⁻³</td>
<td>-</td>
<td>1.5</td>
<td>7</td>
</tr>
</tbody>
</table>

Daudi or Raji cells (10⁶/mL) were mixed with bone marrow (10⁴ nucleated cells/mL), then treated with TiOl IT in the presence of 10 mmol/L NH₄Cl for 24 hours at 37 °C. The percentages of colonies formed per seeded untreated cells were 55% and 25% with Raji and Daudi lines, respectively. *The percentage of cell survival was calculated from untreated controls. Values represent the mean of triplicate wells for each treatment.

Effect of TiOl IT Concentration on T1-Negative Cells Mixed With Human Bone Marrow

Nonspecific toxicity of TiOl IT was examined with the two T1-negative cell lines, Daudi and Raji, which possessed sensitivity to both whole ricin and ricin A-chain that was similar to that of CEM cells (data not shown). One million cells were mixed with bone marrow (10⁵ nucleated cells) and exposed to various concentrations of conjugate. In accordance with the above data, TiOl IT did not harm T1-negative cells up to a 10⁻³ mol/L concentration. Higher doses reduced cell survival (Table 4). Consequently, 10⁻⁴ mol/L could be considered to be the optimal IT concentration.

Absence of Toxicity of TiOl IT for Multilineage Hematopoietic Progenitors (CFU-GEMMT, CFT-GEMM) Derived From Human Bone Marrow

Mononuclear cells from bone marrow samples taken from 11 healthy volunteers were cultured after treatment with various concentrations of IT for multilineage hematopoietic colonies (CFU-GEMMT). The total number of mixed colonies obtained from four individual dishes for each experimental point ranged from 17 to 27. The results of the preincubation with IT are depicted in Fig 4. The treatment of marrow cells with TiOl IT in the absence or presence of 10 mmol/L NH₄Cl did not alter the plating efficiency compared to untreated marrow cells, up to a 10⁻⁴ mol/L concentration, beyond which a slight inhibition was observed. The treatment with A-chain alone, with or without NH₄Cl, gave results similar to those observed with IT (data not shown). Treatment of marrow cells with NH₄Cl alone, or with unconjugated monoclonal TiOl, had no toxic effect. There were no striking differences in the results obtained with eight v 24 hours' preincubation or at 28 °C v 37 °C preincubation temperature and vice versa. Similar dose-response curves were observed for BFU-C (Fig 4) and BFU-E (data not shown).

Identification of T Cells in Mixed Colonies After IT Pretreatment

Individual multilineage colonies were aspirated at day 15 and were examined for the presence of T cells using the peroxidase–antiperoxidase (PAP) slide technique. In a total of 60 mixed colonies derived from IT-treated cells, 23 revealed cells that stained positive for TiOl. The number of positive cells ranged from 0 to 180 cells per colony. The same frequency of mixed colonies containing T cells was observed in these colonies grown from control marrow cells. BFU-E and BFU-C revealed no positive cells for TiOl.

Effect of Cell Concentration

To define the limit of utilization of the conjugate, the influence of the concentration of cells carrying the
T1 antigen was examined in the range of $10^4$ to $10^6$ CEM cells/mL. 99.99% of CEM cells were eliminated, whatever the CEM cell concentration used, up to $10^7$ cells/mL. Only at the highest cell concentration of $10^8$ cells/mL was a 100-fold inhibitory effect observed (Fig 5). Similar results were obtained in the presence of a constant number of bone marrow nucleated cells ($10^7$/mL). The possibility that inhibition might be attributed to an insufficient dose of conjugate due to the increased cell number was ruled out by binding analyses. These studies demonstrated that in all cases, at the doses examined, target antigens were fully occupied (Fig 5). The effect of marrow concentration was further evaluated by adding different numbers of gradient-separated human bone marrow cells to a constant number of CEM cells ($10^6$/mL). Similar inhibition was observed in the presence of $10^8$ bone marrow cells/mL (results not shown). This suggested that high cell density alone could reduce cell sensitivity to the IT. Therefore, it appears that IT treatment should be more effective on unseparated bone marrow, which usually contains around $10^7$ cells/mL, than on concentrated bone marrow cell samples.

**Analysis of IT-Treated Cell Survival**

In order to understand why, under optimal conditions, 0.01% of cells survived the conjugate treatment, we analyzed the surviving cells. Sixty percent of the clones generated after optimal T101 IT treatment (ie, $10^{-8}$ mol/L plus 10 mmol/L NH$_4$Cl for 24 hours at 37 °C) were isolated and cultured for two weeks before analysis. Quantitative determination of the expression of T1 antigen on the cell surface was performed by flow cytometry. Sensitivity to the conjugate was determined by the inhibition of protein synthesis. As shown in Table 5, IT-treated cells that survived did not carry detectable amounts of T1 molecule ($\geq 2,000$), whereas before treatment, they expressed a mean of 20,000 molecules per cell. Surviving cells were highly resistant to the conjugate (Table 5). The possibility that IT treatment had induced antigen modulation was excluded because this phenotype was stable after several weeks in culture (not shown). The possibility that the original CEM cell line could contain an undetectable number of negative cells (less than $1/10^4$) was examined by cloning experiments. The CEM line was subcloned, and we derived CEM1, a cloned cell line, possessing the same average T1 expression and IT sensitivity as the original CEM line (Table 5). Treatment of CEM1 with T101 IT, under the same conditions as before, resulted in a complete eradication of cells from one million added cells, demonstrating that: (1) the limit of IT efficacy in the previous experiment was due to the presence of some negative cells, and (2) all cells expressing the target antigen at a sufficient level could be eliminated with at least a 6-log efficacy factor.

**DISCUSSION**

Syngeneic transplantation of marrow from twin donors has resulted in long-term disease-free survival.

### Table 5. Comparison of Sensitivity to the IT and T1 Expression on Cell Surface of CEM and CEM1 Subcloned Cells Before and After IT Treatment

<table>
<thead>
<tr>
<th>Cells</th>
<th>IT Treatment</th>
<th>Surviving Cells ($10^6$)</th>
<th>IC50 (mol/L)</th>
<th>Average No. of T1 Molecules per Cell*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEM Not treated</td>
<td></td>
<td>$10^6$</td>
<td>$2 \times 10^{-13}$</td>
<td>20,000</td>
</tr>
<tr>
<td>Treated</td>
<td></td>
<td>$4.5 \times 10^2$</td>
<td>$10^{-7}$</td>
<td>2,000</td>
</tr>
<tr>
<td>CEM1 Not treated</td>
<td></td>
<td>$10^6$</td>
<td>$1.8 \times 10^{-13}$</td>
<td>22,000</td>
</tr>
<tr>
<td>Treated</td>
<td></td>
<td>0</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

$10^6$ CEM or CEM1 cells (CEM1 is a subclone of CEM) were exposed for 24 hours at 37 °C to $10^{-8}$ mol/L T101 IT plus 10 mmol/L NH$_4$Cl. Sixty percent of surviving cells that formed colonies were isolated and cultured. These cells were analyzed separately and compared to untreated cells for their sensitivity to the IT estimated by the IC50 concentration that caused a 50% inhibition of 11C-leucine uptake (IC50) and for their expression of the T1 antigen established as described in Materials and Methods. Results are expressed as the mean value obtained from each individual cell population derived from surviving cells that formed colonies.

*The detection limit of this method was around 2,000 molecules per cell.
in 25% to 50% of patients with acute leukemia\textsuperscript{21} and non-Hodgkin’s lymphoma.\textsuperscript{22} These encouraging results have prompted interest in use of autologous bone marrow transplantation for treatment of leukemia and lymphoma when neoplastic cells are eliminated from leukemic marrow.

Various in vitro techniques have been developed, using monoclonal antibodies to eliminate leukemic cells from marrow autografts, such as separation systems using monoclonal antibodies bound to solid phases or cytotoxicity with antibodies plus complement. Recently, another approach has been proposed by Muirhead et al\textsuperscript{23} and Myers et al,\textsuperscript{24} who have shown that immunotoxins can eliminate 99.9% of leukemic cells in human bone marrow with no harm to hematopoietic stem cells, suggesting that this approach could be applied to humans. We have extended these observations with a T cell-reactive IT and have defined conditions for an optimal treatment.

In order to obtain a quantitative measurement of the efficacy of IT in killing leukemia cells, elimination levels were assessed using a reliable and sensitive clonogenic assay. Our results show that the presence of all bone marrow elements did not impair the removal of neoplastic cells with IT. Of the different parameters, only excessive cell concentration leave IT toxicity. For complement-mediated cytotoxicity approaches, a critical cell concentration of 10\textsuperscript{7}/mL also appears.\textsuperscript{25}

Under our optimal conditions (ie, 2 µg T101 IT/mL in the presence of 10 mmol/L NH\textsubscript{4}Cl for eight or 24 hours at 37 °C), a bone marrow graft containing 10\textsuperscript{6} clonogenic T cells/mL, could be rendered completely free of tumor cells, while cells that did not express the relevant target antigen were preserved. This demonstrated the high degree of efficacy and specificity of the hybrid molecule.

Previous studies have shown that the mixture of T101 antibody plus complement, which was cytotoxic against T cells, failed to inhibit the in vitro proliferation of bone marrow and peripheral blood granulocyte-macrophage (CFU-C) or erythroid progenitors (BFU-E).\textsuperscript{5} A lack of toxicity on these precursor cells when the marrow had been treated with T101 IT under the optimal conditions was previously found\textsuperscript{26} and confirmed here. In addition, we extended this study to multilineage hematopoietic progenitors (CFU-GEMMT). The results indicated that pluripotent hematopoietic stem cells were not affected by the T101 IT treatment, suggesting that the stem cell population does not express the T1 differentiation antigen. Furthermore, the treatment of bone marrow cells by T101 IT did not interfere with the repopulation of T-positive T lymphocytes that were observed in mixed colonies after treatment. This might be a very important observation concerning the reconstitution of T cells after IT treatment in autologous bone marrow transplantation. Recently, the removal of leukemic cells from a marrow graft using a monoclonal anti-T cell antibody (anti-Leu-1) of the same specificity as T101 in the presence of complement was described. Such a treatment did not impair hematologic and immunologic reconstitution.\textsuperscript{27} Taken together, these results suggest that T101 IT should not interfere with lymphohematopoietic reconstitution and, therefore, could be useful for autologous bone marrow transplantation in order to purge bone marrow grafts of infiltrated leukemic T cells.

For the elimination of leukemic cells, immunotoxin provides an advantageous alternative to the antibody-plus-complement approach, which requires more manipulations for similar efficacy. Thus, three successive treatments with three monoclonal anti-cALLA antibodies plus complement were required to eliminate 4 logs of leukemic lymphoblasts from the marrow.\textsuperscript{28} In contrast, our findings show that a 6-log reduction was obtained with a single treatment with only one IT. Another advantage of IT is that it is stable over a long time. This is important if it is to be used in therapeutic transplantation trials.

Some laboratories\textsuperscript{29} that have investigated the IT approach were discouraged by the very slow kinetics of cytotoxicity (ie, requiring several days to be effective). We have previously demonstrated that such problems can be easily overcome by using lysosomotropic amines, such as ammonium chloride, at a nontoxic dose. This compound, which likely inhibits IT degradation within the lysosomes, strongly enhances the rate of cell killing of the hybrid, leading to heightened efficiency.\textsuperscript{5,8,29}

The use of an antibody–ricin conjugate is another alternative to improve the kinetics and efficacy of IT cell killing.\textsuperscript{2,30} Such a hybrid requires the presence of lactose to inhibit the nonspecific toxicity induced by the presence of the native binding site of ricin on the B-chain. Precise comparison of A-chain IT plus NH\textsubscript{4}Cl and intact ricin IT plus lactose, using the same antibody on the same cell line, gave identical results.\textsuperscript{31} In our opinion, the low in vivo toxicity (LD\textsubscript{50} in mice, 20 mg/kg)\textsuperscript{31} of the antibody ricin A-chain conjugate is a major advantage because such ITs can be reinfected together with the marrow into the patient. On the other hand, ITs containing whole ricin must be thoroughly eliminated by washing the marrow until nontoxic concentrations are achieved.

Ongoing quantitative studies (Poncelet et al, manuscript in preparation) show that fresh leukemic T1-positive cells coming from T-ALL patients express an average level of T1 (around 10,000 per cell) sufficiently high for IT treatment. However, neoplastic
cells are often heterogeneous in the expression of their cell surface antigens. Therefore, as we have shown in this study, the presence of malignant T1-negative cells or cells that express an extremely low amount of this antigen will escape IT treatment. This may be a limitation of the IT approach, or any other approach using an antibody for tumor cell recognition. A solution to this problem may lie in the use of a cocktail of antibodies that react with various determinants expressed on the neoplastic cell population. However, none of these should harm stem cells.

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REFERENCES


13. Jansen FK, Voisin GA, Baillou CI, Laporte JP, Najman A, Duchateau P: Carayon for FACS analysis is gratefully acknowledged. We thank Drs I Lavabre-Bertrand and J. IaIb for helpful comments and suggestions during the preparation of this manuscript. We thank Drs P. Capel and D. Vallera for providing us the bone marrow samples. The expert technical assistance of P. Carayon for FACS analysis is gratefully acknowledged. We also wish to thank Dr H.E. Blythman for English corrections and A. Garcia for typing the manuscript.


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