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Donor bone marrow treatment with T101 Fab fragment-ricin A-chain immunotoxin prevents graft-versus-host disease

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Summary:

Thirty-eight patients with haematological malignancies were treated with bone marrow transplantation using histocompatible immunotoxin T cell-depleted marrow siblings. All patients received conventional postgraft immunosuppression (methotrexate and/or cyclosporin A). Donor bone marrow was treated *ex vivo* with T101 Fab fragment coupled to ricin A-chain (T101 Fab-RTA) at a concentration of 10^{-8} M of A-chain in association with NH_4Cl (2×10^{-2} M) in pH adjusted (7.8) incubation medium. A median cytoreduction of 99.5% (91–99.5) was obtained. The median of follow-up was 300 days. Only three patients developed grade II acute graft-versus-host disease (GVHD) (actuarial rate of acute GVHD: 9.1%). No chronic GVHD occurred. All patients but one engrafted. Six out of the 37 patients developed a documented bone marrow rejection (actuarial rate of graft failure: 18%). Ten patients relapsed (actuarial rate of relapse: 36.9%). These findings demonstrate that treatment of donor marrow with T101 Fab-RTA in association with NH_4Cl at critical pH value can achieve a high level of mature T cell depletion and greatly reduce the incidence of bone marrow rejection and relapse after T cell-depleted allogeneic bone marrow transplantation.

Acute graft-versus-host disease (GVHD) remains a major cause of morbidity and mortality after allogeneic bone marrow transplantation (BMT) despite the use of active immunosuppressive drugs such as methotrexate[1] or cyclosporin A,[2] alone or in combination.[3] A number of experimental studies showed that the severity of acute GVHD could be greatly reduced if a sufficient number of mature T cells were eliminated from the donor bone marrow before transplantation.[4] Various methods have been used to remove mature T cells from donor bone marrow in a clinical situation to prevent GVHD.[5–14] These studies, in a large series of patients grafted from fully HLA-identical siblings, showed a favourable effect of mature T cell depletion on the incidence and severity of both acute and chronic GVHD while an unexpected, relatively high, although variable, incidence of bone marrow rejection[10,11,13,15] and relapse was observed.[11,14,15]

In order to simplify and standardize the bone marrow purging procedure, immunotoxins (ITs) represent an interesting approach. The use of ricin A-chain ITs seems more advantageous than whole ricin ITs[16] since neither lactose pre-incubation nor extensive washing are needed. In addition, the efficacy of ricin A-chain IT is comparable, if not superior, to the whole ricin molecule when the former is used in combination with lysosomotropic amines such as NH_4Cl as enhancers.[17]

In preclinical studies, we found that T101 whole Ig-RTA (T101 Ig-RTA) could induce, in the presence of NH_4Cl (10^{-2} M), a cytoreduction of mature T cells higher than 99%[18,19] while haematopoietic progenitor stem cells were unaffected.[20] Subsequently, a first series of patients with haematological malignancies were grafted from histocompatible donor bone marrow treated *ex vivo* with T101 Ig-RTA. Surprisingly, this study showed non-reproducible and generally poor T cell cytoreduction with a high incidence of both severe acute and chronic GVHD (unpublished results). Further studies identified the pH of the incubation medium as a critical factor which could explain the discrepancy between poor clinical results and laboratory findings.[21] Moreover, these studies showed that T101-Fab fragment coupled to ricin A-chain (T101 Fab-RTA) displayed a lesser dependence from pH and a higher mature T cell cytoreduction[21] despite its lower affinity.[22]

Therefore another clinical study was designed by using T101 Fab-RTA (10^{-8} M) in association with NH_4Cl (2×10^{-2} M) in pH-adjusted (7.8) incubation medium. Thirty-eight patients entered into this study. A median cytoreduction of 99.5% could be obtained and no patient developed acute GVHD greater than grade II or chronic GVHD.

Materials and methods

Preparation of immunotoxins

Purified whole monoclonal T101 IgG antibody (T101 IgG) was purchased from Hybritech Inc. (La Jolla, CA).

To prepare T101 Fab-RTA, T101 antibody was digested with papain to produce monovalent Fab molecules. Antibody fragments were then purified by ion

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exchange chromatography. Analysis of Fab fragment by SDS-polyacrylamide gel electrophoresis revealed undetectable levels of whole antibody and less than 0.5% Fc fragment contamination. T101 Fab fragments were coupled to ricin A-chain in our laboratory (Sanofi Recherche, Montpellier, France) according to the method previously described.[23]

Evaluation of mature T cell cytoreduction by FACS analysis

Measurement of T cell depletion was performed according to the technique previously reported.[18] Briefly, after treatment with T101 Ig-RTA or T101 Fab-RTA, gradient density separated bone marrow mononuclear cells were washed and then seeded in 2 ml macrowell plates (Linbro-Flow Laboratories) at a final concentration of 5×10^5 cells/ml in the presence of 0.5 U/ml of interleukin 2 (IL2) (provided by Dr Fradelizzi, IGR, Villejuif, France) and 1% phytohaemagglutinin (PHA) (Wellcome, Dartford, UK). Fluorescence activated cell sorter (FACS) analysis of stimulated T lymphocyte growth kinetics was performed at different times (48, 72 and 96 h by measuring viable T cells stained by indirect immunofluorescence using a panel of anti-T monoclonal antibodies (MoAbs) (CD5, CD2, CD3, CD4 and CD8) and FITC-F (ab') 2 sheep-antimouse Ig. Cytofluorometric studies were carried out on living cells using PLS and FITC parameters, both in logarithmic amplification. Viable T lymphocytes were counted, with non-fluorescent beads (250 000 beads per well) as internal standard. Cytoreduction was evaluated by calculating the ratio between the absolute number of viable T cells in treated samples and in the control group. Cytoreduction was expressed as a percentage of controls. The sensitivity of the assay was evaluated at 3 logs (99.9%).[18] Measurement of mature T cell depletion was performed in a single laboratory (Sanofi Recherche, Montpellier). All 38 treated bone marrows could be evaluated.

Design of the study

Marrow treatment procedure Donor bone marrow mononuclear cells were obtained from Ficoll–Hypaque gradient density separation using an IBM 2991 blood cell processor. Mononuclear cells were resuspended in RPMI 1640 medium supplemented with 5% (w/v) final concentration human serum albumin (HSA) at 2×10^7 cells/ml in sterile plastic bags. The pH of incubation medium was adjusted to 7.8 by the addition of Thamacetat (Roger Bellon, France) used at a standard final concentration of 5% (v/v). Marrow cells were then treated with T101 Fab-RTA (10^{-8} M expressed in A-chain content) in the presence of NH_4Cl (2×10^{-2} M) at 37°C for 2 h. Treated marrow was directly reinfused without washing.

Patient characteristics The 38 patients included into this study were suffering from haematological malignancies. All the patients were grafted from fully HLA-matched MLC-negative sibling donors and received conventional post-graft immunosuppression (methotrexate and/or cyclosporin A). The characteristics of patients are detailed in Table I. The median age was 34 years (range 5–43). Nineteen patients had chronic myeloid leukaemia (CML), nine patients had acute myeloid leukaemia (AML), six patients had acute lymphoblastic leukaemia (ALL), two patients had a malignant lymphoma and two patients were transplanted for a preleukaemic state. At the time of BMT, 23 patients were in 1st complete remission (CR 1) of acute leukaemia or in 1st chronic phase of CML (CP 1), five were in relapse, ten were grafted after the 1st CR of acute leukaemia or in accelerated phase of CML. Preparative regimens consisted of high doses of alkylating agents (cyclophosphamide 60 mg/kg for 2 days in 32 patients, melphalan 140 mg/m^2 single dose in six patients) followed by total body irradiation (TBI) as a single dose (1000 cGy in 23 patients) or fractionated (1200 cGy in 15 patients). Four patients received in addition to single dose TBI, a total nodal irradiation (TNI) (800 cGy). Since we were exploring the clinical efficiency of a new T cell-depletion technique, conventional preventive treatment of GVHD was administered systematically. Post-graft immunosuppression was different from one centre to another and consisted of (1) methotrexate (13 patients), (2) cyclosporin A (8 patients), or (3) both (17 patients).

Supportive care included patient isolation in laminar air-flow rooms, and prophylactic transfusions of irradiated red blood cells and platelets. GVHD was coded as previously described.[1] Probabilities of acute GVHD, relapse, survival and disease-free survival were evaluated using the method of Kaplan–Meier.

Eight French bone marrow transplant centres (members of the GEGMO: Groupe d'Etude de la Greffe de Moelle Osseuse) took part in this study. The protocols were approved by the Review Boards of the French Cooperative Group of Bone Marrow Transplantation and of each participating centre as well as by the review board of Sanofi.

Results

After donor bone marrow treatment with T101-Fab-RTA and NH_4Cl, mature T cell cytoreduction varied from 91 to 99.9% with a median cell kill of 99.5%. In 32 cases, T cell depletion was higher than 99%.

Initial engraftment occurred in 37 out of the 38 patients. The median number of days for granulocytes to reach 500×10^6/l and platelets 50×10^9/l were 25 and 35 respectively.

Only three patients developed a Grade II acute GVHD (see Figure 1). In each case T cell depletion was higher than 99.0%. No patient developed chronic GVHD (median follow-up of 200 days).

Six patients developed a documented bone marrow rejection (actuarial probability of rejection = 18%) which occurred between days 25 and 140 (median = 65 days) (Figure 2). From these patients, two died, two relapsed and two are still alive in a chronic phase of their diseases (CML and preleukaemic state respectively). Thus, a total of seven patients developed graft failure (no take or bone

Table I Patient characteristics and clinical outcome

Number of patients	38
Median age (years)	34 (5–43)
Age ⩾ 30	24
Diagnosis	
CML	19
AML	9
ALL	6
Lymphoma	2
Preleukaemic state	2
Clinical status at time of BMT	
Relapse	5
CR >1 or AP of CML	10
CR1 or 1st CP of CML	23
Conditioning regimen	
Cyclophosphamide/Melphalan-TBI	32/6
TBI 1000 cGy × 1	23
TBI fractionated 1200 cGy	15
Prevention of GVHD	
Methotrexate (MTX)	13
Cyclosporin A (CSA)	8
MTX-CSA	17
Engraftment Initial engraftment (n/total):	37/38
Median dose of reinfused nucleated cells ($\times 10^8$/kg):	0.93 (0.4–4)
Median dose of reinfused CFU-GM ($\times 10^4$/kg):	24.8 (0.75–44.8)
Median day for granulocytes 500 × 10^6/l	25 (12–40)
Median day for platelets 50 × 10^9/l	35 (13–141)
Median cytoreduction (%):	99.5 (91–99.9)
Acute GVHD ⩾ II Number (*):	3 (9.1%)
Chronic GVHD Number (*):	0 (0%)
Graft failure Number (*):	7 (18%)
Relapse Number (*)	10 (36.9%)
Survival Number (*):	23 (54.8%)
Disease free survival Number (*):	15 (37.7%)

(*) = actuarial rate
CML = chronic myeloid leukaemia
AML = acute myeloid leukaemia
ALL = acute lymphoblastic leukaemia
CR1 = first complete remission
TBI = total body irradiation
CP = chronic phase
AP = accelerated phase

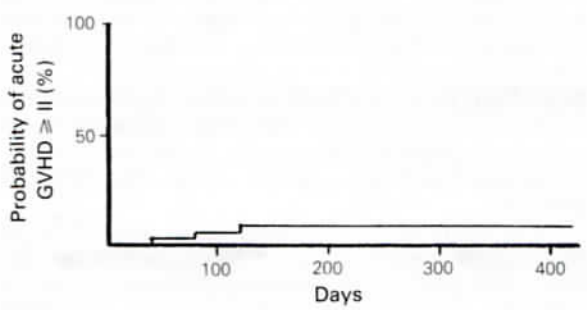

Figure 1 Probability of developing acute GVHD of grade II or greater for 38 patients transplanted for haematological malignancy.

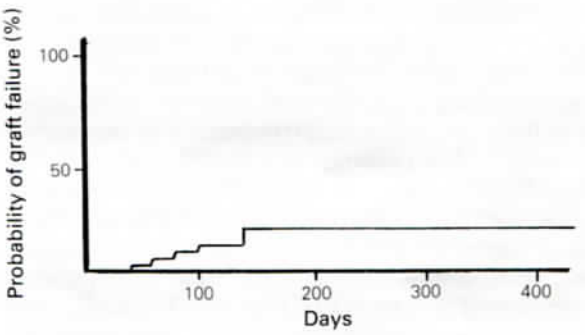

Figure 2 Probability of developing graft failure for 38 patients allografted for haematological malignancy.

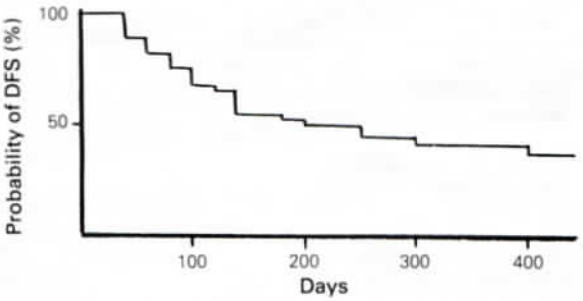

Figure 3 Probability of disease-free survival (DFS) for 38 patients transplanted for haematological malignancy.

marrow rejection). Those seven patients were conditioned either with unfractionated 1000 cGy single dose TBI (four cases) or fractionated 1200 cGy TBI (three cases). Two patients received in addition to a single dose TBI, a total nodal irradiation (800 cGy). No correlation between the incidence of bone marrow rejection and reinfused CFU–GM dose was found. Patients who did not reject the graft were conditioned with either unfractionated (19 cases) or fractionated TBI (12 cases).

Ten patients relapsed, four patients had high risk clinical status (grafted in refractory leukaemic phase and in CR2 or subsequent complete remission). Four patients were grafted in CR1 (two common ALL, one AML M5 and one ALL L3 FAB subtype). Two patients were grafted in accelerated phase of CML. Relapses occurred between days 60 and 356 (median = 140 days). The Kaplan–Meier estimation of relapse was 36.9%.

Twenty-three patients are still alive (probability of survival: 54.8%). Fifteen are disease-free surviving (probability DFS: 37.7%) (Figure 3). The median of follow-up of the study was 300 days (23–627).

Discussion

The present study confirms that donor marrow treatment with T101 Fab-RTA (10^{-8} M) in the presence of NH_4Cl (2×10^{-2} M) for 2 h at 37°C and at pH 7.8 is a reproducible, efficient, reliable, safe and simple T cell-depletion technique. Further studies showed that this method could still be simplified by using buffy coat preparations, with a similar level of T cell cytoreduction.[24] In a series of 38 patients, the majority had high risk of GVHD, the incidence of both acute and chronic severe II–IV GVHD was greatly reduced (actuarial rate of 9.1%). Our results are in line with those reported by other groups using T cell-depleted marrows in allogeneic matched BMT for leukaemia, irrespective of the methodology used.[8–13] For example, in a randomized study the French GEGMO cooperative group showed an overall incidence of severe grade III–IV acute GVHD of 42% in the non-depleted control group treated with methotrexate and cyclosporin A while only three out of 47 patients (6%) developed severe GVHD in the T cell-depleted group using a cocktail of monoclonal antibodies and rabbit complement.[15]

However, in our study a relatively high rate of graft failure was observed; six patients rejected their marrow and one patient failed to engraft (actuarial rate: 18%). Bone marrow rejection after T cell-depleted BMT has previously been reported.[10,11,13] In a randomized study using a cocktail of MoAbs and rabbit complement, the GEGMO cooperative group showed an overall incidence of graft failure (no take or rejection with bone marrow failure) as high as 23% while no documented rejection was observed in the non-depleted control group.[15] Our study as well as the GEGMO study, showed a higher incidence of bone marrow rejection than that reported by most other groups using T cell depletion. It is possible that post-transplantation immunosuppressive therapy had influenced unfavourably the rate of graft failure. The incidence of relapse in our study was relatively higher (37%) than that previously reported after T cell depletion, probably due in part to the fact that most patients presented with high risk haematological status (acute leukaemia in 2nd–3rd remission or in refractory phase). On the other hand, the incidence of relapse in our standard risk patients may still increase with a longer follow-up as suggested by other studies.[14,15] Therefore, it is difficult to draw any conclusion about the impact of T101 Fab-RTA T cell depletion on relapse risk.

Finally, this study showed that donor marrow treatment with T101 Fab-RTA is an excellent alternative to other immunodepletion techniques for prevention of GVHD. However, the high incidence of graft failure raises the question of additional treatment of recipients to achieve stable engraftment. In that respect the possible use of immunotoxins active *in vivo*[25] could be a very attractive alternative to other specific immunosuppressive therapy of recipients. Until such therapeutics become available, T cell depletion alone, in standard risk patients, remains questionable.

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