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IL TRAPIANTO DI CELLULE STAMINALI EMATOPOIETICHE NELLA PATOLOGIA PEDIATRICA ONCOEMATOLOGICA ED AUTOIMMUNE

Trieste, 24-25 novembre 2000
IRCCS Burlo Garofolo

Editors
M. Andolina, G. Dini. C. Uderzo

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IL TRAPIANTO DI CELLULE STAMINALI EMATOPOIETICHE NELLA PATOLOGIA PEDIATRICA ONCOEMATOLOGICA E AUTOIMMUNE

IRCCS BURLO GAROFOLO, TRIESTE
24-25 NOVEMBRE 2000, MUSEO REVOLTELLA

VENERDI' 24 NOVEMBRE

ore 9 **APERTURA DEL CONGRESSO**
E. Arbustini
Direttore Scientifico
IRCCS Burlo Garofolo, Trieste

1° SESSIONE: TRAPIANTO ALLOGENICO
Moderatore F. Locatelli (Pavia)

ore 9.30 Indicazioni in ematologia
P. Tamaro (Trieste)

ore 9.50 Il trapianto nella LLA in prima remissione
C. Uderzo (Monza)

ore 10.30 Trapianto non mieloablativo in pediatria
R. Miniero (Torino)

ore 10.50 *Coffee break*

2° SESSIONE: IL PROBLEMA DEI DONATORI
Moderatore R. Miniero (Torino)

ore 11.20 Caratteristiche biologiche delle cellule staminali cordonali
V. Rosti (Pavia)

ore 11.40 Esperienze cliniche in pediatria
F. Locatelli (Pavia)

ore 12.00 Il trapianto da donatore volontario
G. Dini (Genova)

ore 12.20. Il trapianto aploidentico
M. Andolina (Trieste)

ore 12.40 Il trapianto aploidentico nelle immunodeficienze
F. Porta (Brescia)

Pranzo

ore 14.30 TAVOLA ROTONDA
Consensus sulla terapia della LLA in seconda remissione
F. Locatelli, G. Dini, C. Uderzo, G. Basso, P. Tamaro, M. Andolina

ore 15.45 *Coffee break*

3° SESSIONE: TERAPIA DI SUPPORTO
Moderatore P. Tamaro (Trieste)

Medici ed infermieri. Opinioni a confronto su:

ore 16.00 Profilassi e terapia delle infezioni nel trapiantato
E. Gombach (Trieste), G.A. Zanazzo (Trieste)

ore 16.30 Diagnostica virologica
P. D'Agaro (Trieste)
P. Grossi (Pavia)

ore 17.00 Nutrizione parenterale e gestione del CVC
M. Candusso (Cosenza)
R. Guerrato (Trieste)

ore 17.30 Strategie trasfusionali
G. De Silvestro (Padova)
S. Parco (Trieste)

Discussione

SABATO 25 NOVEMBRE

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Moderatore A. Ventura (Trieste)

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S. Dall'Orso (Genova)

ore 9.30 Indicazioni nelle malattie autoimmuni
A. Marmont (Genova)

ore 10.00 Trapianto non mieloablativo: l'esperienza di Trieste
M. Rabusin (Trieste)

ore 10.20 Deplezione funzionale dei linfociti
G. Presani (Trieste)

ore 10.30 Autotrapianto vs. trattamenti alternativi in reumatologia pediatrica
L. Lepore (Trieste)

ore 11.00 *Coffee break*

ore 11.30 Trapianto si, trapianto no nelle malattie autoimmuni: casi clinici commentati dall'esperto
A. Marmont (Genova), A. Martini (Pavia), F. Zulian (Padova), F. Falcini (Firenze), R. Cimaz (Milano), M. Gattorno (Genova)

ore 13.30 *Chiusura del congresso*

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HEMATOPOIETIC STEM CELL TRANSPLANTATION IN PEDIATRIC ONCOLOGIC-HEMATOLOGIC AND AUTOIMMUNE DISEASES

Trieste, November 24-25, 2000, IRCCS "Burlo Garofolo"

The history of pediatric bone marrow transplantation began in 1984. From then to December 1999 20 teams performed more than 2800 autologous and allogeneic transplants. The members of the *Italian Pediatric Association for Haematology and Oncology* (AIEOP) were the first in Italy to organize a Co-operative Group.

In 1984 both autologous and allogeneic transplants were performed in leukemia and solid tumors; in 1986 we performed the first haploidentical bone marrow transplantation (BMT), in 1989 the first matched unrelated donor (MUD) BMT, in 1994 the first cord blood bone marrow transplantation. In 1994 even autoimmune diseases became an indication for autologous bone marrow transplantation.

This huge activity led to several cooperative studies, publications and meetings. This meeting will try to give an overview of the outstanding results of the Erice meetings.

We will focus on the questions that need an answer in year 2000:

- indications for BMT, focusing on the new ones and on the ones that to day seem obsolete
- reduction of toxicity of conditioning regimens;
- stem cells sources others than HLA identical sibling: MUD, cord blood, haploidentical donors
- consensus on the most important indication for BMT: acute lymphoblastic leukemia in second complete remission;
- autoimmune diseases: immunoablation with or without stem cell rescue;
- supportive therapy: in cooperation with the nurses we will try to design common protocols of prevention and treatment of infections, of parenteral nutrition and transfusion strategy.

The spectrum of diseases curable by hematopoietic stem cell transplantation is getting larger, although the decision whether to offer a transplant or alternative therapy can be difficult because of so many factors which can affect with short and long-term outcome and quality of life of the patients.

The AIEOP Group, by accumulating information from multicenter studies and the national Registry, is now able define the priorities for future recommendations in the care of patients with hematologic malignancies or disorders.

Finally, new research trends need to be defined, not only in the clinical setting but also in immunobiology and all other fields of interest, in order to improve the management and results of the BMT procedures.

the editors
M. Andolina, G. Dini, C. Uderzo

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original paper

Allogeneic bone marrow transplantation in hematologic disorders of childhood: new trends and controversies

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ABSTRACT

Bone marrow transplantation (BMT) represents an important therapeutic choice for several kinds of disorders: hematologic, metabolic and neoplastic pathologies can be treated with this strategy. The aim of this article is to describe the main indications for allogeneic BMT in haematologic disorders of childhood and possible problems related to this procedure. We consider only hematologic aspects, paying particular attention to unusual disorders of infancy as myelodysplastic syndromes and aplastic anemia. We also consider quality of life after a BMT in patients with sickle cell anemia and thalassemia major and compare this with quality of life of patients receiving chronic periodic blood transfusions.

Key words: allogeneic bone marrow transplantation, hematologic disorders, myelodysplastic syndromes, severe aplastic anemia, childhood, pediatric, infancy, thalassemia major, ALL, HR-ALL, sickle cell anemia, AML, CML

Bone marrow transplantation (BMT) has become the treatment of choice for several forms of malignant and non-malignant hematopoietic disorders, solid tumors, inborn errors of metabolism and immunodeficiency states. The aim of this article is an analysis of new trends in the use of allogeneic BMT in hematologic diseases of childhood and possible problems related to this procedure.

Table 1 lists the hematologic disorders of childhood that can be treated by allogeneic BMT. The major indication is malignant disease of the bone marrow, but a wide range of non-malignant conditions may also be cured by allograft procedures with varying degrees of success: for some of them allogeneic BMT has already become the therapeutic method of choice, for some others there is still no agreement.

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Allogeneic bone marrow transplantation in acute lymphoblastic leukemia

The considerable progress of chemotherapeutic regimens has improved the prognosis of paediatric ALL, resulting in a 5-year percentage of survival of 60-80%.[1-3] Allogeneic stem cell transplantation represents a therapeutic choice for very high risk (HR) patients or when chemotherapy fails to achieve a permanent remission: prognosis and quality of life of grafted children has also been improving in recent years, mostly thanks to better supportive management.[1-5]

Tables 2 and 3 list, in order, the indications for allogeneic transplant and the criteria necessary to assign a patient to the HR-group of treatment, according to the latest therapeutic protocol of the AIEOP (ALL 2000).

High risk acute lymphoblastic leukemia (HR-ALL)

In these patients chemotherapy alone can induce a complete remission (CR) in 30-40% of cases.[4,5] Some retrospective studies demonstrate that more than 60% of children with high risk acute lymphoblastic leukaemia (HR-ALL) in first remission can recover thanks to HLA-identical sibling BMT.[6-9]

As far as concerns the indications for an unrelated donor marrow transplant in children with HR-ALL in first CR, there is still no consensus. Though the few data we have at our disposal are encouraging (total survival 60%),[10,11] new studies on larger series are needed to evaluate the efficacy and the advantages of this procedure as compared with chemotherapy.

Acute lymphoblastic leukemia in second or subsequent remission

Allogeneic BMT from an HLA-compatible donor is frequently considered the treatment of choice in children who achieve a second or subsequent remission after marrow relapse. In fact, the probability that rescue chemotherapy fails is increased by the highly intensive protocols utilized for primary and relapse therapies.

Table 1. Indications for allogeneic BMT in pediatric hematopoietic disorders.

MALIGNANT HEMATOLOGIC DISORDERS	
LEUKEMIAS AND LYMPHOMAS	
1. Acute lymphocytic leukemia in first complete remission in very high risk patients	
2. Acute lymphocytic leukemia in second or subsequent complete remission	
3. Acute myelogenous leukemia in first complete remission or in first relapse	
4. Chronic myelogenous leukemia-Ph+	
5. Hodgkin's lymphomas after second complete remission	
6. Non-Hodgkin's lymphomas after second complete remission	
MYELODYSPLASTIC SYNDROMES	
NON-MALIGNANT HEMATOLOGIC DISORDERS	
CONGENITAL	1. HEMOGLOBINOPATHIES: Sickle cell anemia, Homozygous β-thalassemia
	2. Fanconi's anemia
	3. Dyskeratosis congenita
	4. Congenital amegakaryocytosis
	5. Wiskott-Aldrich syndrome
ACQUIRED	1. Severe aplastic anemia
	2. Paroxysmal nocturnal hemoglobinuria

Table 2. AIEOP indications for allogeneic stem cell transplantation in ALL.

HLA-IDENTICAL SIBLING DONOR
1. Non-achievement of a complete remission (CR) by 33rd day
2. Prednisone poor responders (PPR) with T or pre-pre-B immunophenotype or with >100,000/mm³ leukocytes
3. Presence of translocation t(9;22) or BCR/ABL
4. Presence of translocation t(4;11) or MLL/AF4
5. Minimum residual disease (MRD) on 78th day ≥10-3
HLA-IDENTICAL UNRELATED DONOR
1. Non-achievement of a complete remission (CR) by 33rd day
2. PPR with t(9;22) or BCR/ABL
3. PPR with t(4;11) or MLL/AF4
4. MRD at 78th day ≥10-2

Table 3. AIEOP criteria to assign patients to the HR-group of treatment. Presence of one criteria is sufficient for the assignation.

1. Prednisone poor responders (PPR): inadequate response to pre-phase treatment (7 days) with prednisone (and with IT-methotrexate at day 1): ≥1,000 leukemic cells/μL of peripheral blood on 8th day.
2. Non-achievement of a complete remission (CR) by 33rd day.
3. Presence of translocation t(9;22) or t(4;11)
4. Minimum residual disease (MRD) ≥ 10-3 on 8th day.

Children who relapse while receiving chemotherapy have a long-term disease-free survival (DFS) of less than 10%, and those who relapse more than 1 year off therapy have a DFS of 30%. Patients who receive a marrow transplant from an HLA-identical sibling or unrelated donor have a DFS of 40-50%. For patients who undergo allogeneic BMT from an HLA-identical sibling, the 5-year event-free survival (EFS) for ALL is 75% in 1st CR, 60.4% in 2nd CR, and 22.3% in > 2nd CR. Moreover, if relapse occurs over 5 years since the onset of disease, the advantages of allogeneic BMT compared to chemotherapy become statistically non-significant (LFS: 55% versus 30%).[6,7]

Allogeneic bone marrow transplantation in acute myelogenous leukemia (AML)

Allogeneic BMT is considered the treatment of choice for children with acute myelogenous leukemia (AML) who have an HLA-identical related donor. In the last 25 years several retrospective and prospective studies reported the efficacy of allogeneic BMT in childhood AML during first complete remission and demonstrated its superiority over intensive chemotherapy, autologous BMT and conventional maintenance chemotherapy.[8-10] A prospective study published in 1991 by the AIEOP reported that EFS was obtained in 64% of children with AML in first CR treated with allogeneic BMT, in 50% of those treated with an autologous marrow transplant and in 35% of those who received chemotherapy alone.[11] Most of the following studies substantially confirmed these data. In spite of this, the results of transplant procedures for AML must always be compared with results of contemporary chemotherapy regimens; some groups, such as the BFM group, discourage allograft procedures for patients in first remission with cytogenetically "favorable" sub-types of AML.

However, after marrow relapse AML can become chemoresistent with a survival percentage of 20%: it is therefore appropriate to perform allogeneic BMT in all patients with AML in first CR who have a related HLA-compatible donor available.

Data reported by Bacigalupo[12] indicate that AML can be cured with allogeneic BMT after conditoning regimen (TBI 9.9 Gy, cyclophosphamide 120 mg/kg) and low dose immunosuppression (CyA 1 mg/kg): patients treated with this regimen had a 5-year relapse rate significantly better as compared with patients treated with TBI < 9.9 Gy and CyA 5 mg/kg post graft.[12]

For the 70% of patients who do not have a suitable matched family donor available, other

sources of hematopoietic stem cells include unrelated donors and autologous marrow. The results of BMT from unrelated donors demonstrate a 35% of DFS at 8 years. The search for an unrelated donor can be very long, so, when a related donor is not available, allogeneic BMT should be considered just as if chromosome alterations are present or in patients who do not respond to chemotherapy. Otherwise, transplants involving unrelated donors for AML in remission should proceed largely in the context of a clinical research protocol.[13] As far as regards autologous BMT, studies by both the Children Cancer Group and the Pediatric Oncology Group have not shown any difference in DFS between children receiving autologous transplant and those receiving continued chemotherapy (50% and 44%, respectively for the CCG study; 38% and 36% for the POG study).[12,13]

Allogeneic bone marrow transplantation in chronic myelogenous leukemia

Allogeneic or syngenic BMT is an effective treatment for chronic myelogenous leukemia (CML);[17] the results are dependent on the phase of the disease at the time of BMT. Allogeneic grafting should ideally be performed during the chronic phase within 1 year from diagnosis, but patients in advanced phases may also be treated with this procedure on an individual basis. Children who receive a transplant during blast crisis have a 10% to 20% DFS, those in accelerated phase have a 35% to 40% DFS, and those in chronic phase have a 50 to 80% DFS. Among chronic phase patients the major factor predicting outcome is the length of time between diagnosis and transplant:[17-18] those who receive a transplant within the first year after diagnosis have a DFS of 80-85% in the first 3 years, whereas those who receive a transplant more than 2 years after diagnosis have a DFS of 50-60%.[19]

Unrelated donor marrow transplants give results similar to those obtained from suitably matched family member donors but are usually performed in the context of clinical research protocols.

Allogeneic bone marrow transplantation in myelodysplastic syndromes

Myelodysplastic syndromes (MDS) (Table 4) represent a heterogeneous group of clonal alterations of hematopoiesis, having in common cytopenia, dysplasia and a predisposition to evolve in acute myeloid leukemia (20-30% of cases). These disorders are typical of adult age: they represent only 2-9% of childhood hematologic malignancies; furthermore MDS of childhood appear to run a more aggressive course, especially forms with elevated numbers of blasts in marrow (primary MDS).[15,20,21] Several conditions have been identified as predisposing factors for childhood MDS and are reported in Table 5.

Table 4. Myelodysplastic syndromes in children.

PRIMARY MYELODYSPLASIA	
	Refractory anemia (RA)
	Refractory anemia with ring sideroblasts (RARS)
	Refractory anemia with excess of blasts (RAEB)
	Refractory anemia with excess of blasts in transformation (RAEB/T)
	Juvenile chronic myeloid leukemia
	Congenital myelodysplastic sindrome
SECONDARY MYELODYSPLASIA	
	Familial
	Therapy induced

Table 5. Constitutional abnormalities and somatic derangements associated with MDS in children.

- Down's syndrome
- Trisomy chromosome 8
- Monosomy chromosome 7
- Ataxia-teleangectasia
- Bloom's syndrome
- Xeroderma pigmentosus
- Nijmegen's syndrome
- Neurofibromatosis I
- Schwachman's syndrome

Refractory anemia, refractory anemia with ring sideroblasts and refractory anemia with excess of blasts in transformation

Refractory anemia (RA) is frequent in children with familial hematologic disorders or constitutional problems. Refractory anemia with ring sideroblasts (RARS) is the least common of all the group: typically it is observed in girls with constitutional abnormalities and karyotype alterations (frequently monosomy 7); it can be a genetic mitochondrial disorder with polyclonal hematopoiesis that seldom progresses to leukemia but rather terminates in multiorgan failure; few cases of spontaneous regressions are described. Refractory anemia with excess of blasts (RAEB) and particularly with excess of blasts in transformation (RAEB/T) can be observed in older children: these forms are more aggressive than other myelodysplasias because of a higher progression rate to AML and poor survival.

Allogeneic BMT performed early in the course of the disease and from the most closely matched source available confers the best hope for long-term survival. Some series record a dis-

ease-free survival for childhood MDS as high as 60% following allogeneic transplantation. Patients transplanted for RA, RARS, RAEB, RAEB/T have a 5-year DFS of 52%, 34%, 19 % and 26%, respectively. The 5-year overall survival (OS) for the respective patient groups was 57%, 42%, 24% and 28%. In a multivariate analysis, younger age, shorter disease duration and absence of excess of blasts were associated with improved outcome.[20]

Down's syndrome was the most common affinity reported and one which is significantly associated with RAEB. As far AML, so far MDS in children with Down's syndrome (or other constitutional alterations), the outcome is unfavorable. Moreover BMT for this group of patients appears to be associated with early mortality and to offer no advantages over conventional chemotherapy or supportive management.[16,18]

Juvenile monocytic myeloid leukemia or juvenile chronic myeloid leukemia

Juvenile chronic myeloid leukemia (JMML) is typical of very young patients (median 2.6 years old): it is characterized by prominent hepatosplenomegaly, frequent skin involvement, leukocytosis, monocytosis and presence of immature precursors in the peripheral blood. It is frequently associated with neurofibromatosis.[1] About 65% of patients with JCML have a normal karyotype, while 25% have monosomy 7 and 10% a complex of abnormal alterations.[19-22]

Some young children with monosomy 7 karyotype share many of the clinical, laboratory and pathological features of JMML. Several study groups have attempted to classify this group of young children into a separate disorder called monosomy 7 syndrome. These patients did initially appear to differ from others with JMML because of a lower fetal hemoglobin level, and longer survival but higher rate of transformation to AML. However, more recent studies suggest that there are no data to support the concept of monosomy 7 as a truly separate disorder, but rather that it should be considered as a cytogenetic opportunist.[22]

In a large series of patients with JMML the median survival without bone marrow transplantation was 1 year. Prognostic factors for duration of survival without BMT are platelet count at the time of diagnosis, age and HbF: 70% of patients aged < 2 years; platelets > 33,000/mL and HbF < 15% are still alive 3 years after diagnosis. Allogeneic BMT is the only therapy that has thus far produced unequivocal sustained remissions. The major limiting factor is an inordinately high percentage of relapse; allograft procedures can offer a 43% of 5-year survival.[19,21,22]

Allogeneic bone marrow transplantation in severe aplastic anemia

Aplastic anemia (AA) is a physiologic and anatomic failure of bone marrow's with a marked decrease or absence of blood forming elements. It is characterized by peripheral pancytopenia without hepatomegaly, splenomegaly or lymphoadenopathy. The clinical presentation is related to the severity of the pancytopenia. The overall incidence is 2 to 4 cases per million children <15 years old in the world.

International criteria for diagnosis of the severe forms are listed in Table 6.

Table 6. International criteria for the diagnosis of severe aplastic anemia.

1. polymorphonuclear neutrophils $<0.5 \times 10^9$/L
2. platelets $<20 \times 10^9$/L
3. retilculocytes < 1%
4. bone marrow cellularity <30% normal

Failure of hematopoiesis in children may be congenital (Fanconi's anemia, dyskeratosis congenita) or acquired (viruses, drugs, environmental toxins). Causes remain unknown in more than 70% of cases (idiopathic SAA). Hypothetical mechanisms of idiopathic SAA include a primitive stem cell defect, a stromal defect or an autoimmune process directed against marrow progenitor cells: the last possibility is supported by the clinical response to immunosuppression. There is noresponse to stem cell growth factors in any case.[23]

SAA has an overall mortality rate of 80% if managed with supportive care alone. Before the advent of BMT and immunosuppressive therapy (IST) the median survival was 10 to 20 months for patients and was related only to supportive treatment. Use of immunosuppressants, especially antilymphocyte globulin, have improved the prognosis of those patients who cannot receive a BMT from an HLA-identical sibling: beneficial effects of IST appear to depend primarily on the cause of SAA.[23-26]

A recent analysis by the EBMT of 1,182 children treated with IST has shown a significant improvement in survival from 58 to 75% at 5 years over the last two decades. These patients, in contrast to those given a bone marrow transplantation,are not considered cured because of the high rate of relapse and development of clonal disease (10% and 30%, respectively).[23-26]

Allogeneic BMT for severe aplastic anemia was introduced in the early seventies and is still considered the first-line therapy for young patients. Recently the EBMT analyzed a large group of

patients grafted in Europe from 1976 to 1998 and demonstrated that factors predicting outcome of those patients are: age at time of graft, donor type, interval between diagnosis and BMT, year of BMT and female donor to male recipient. Patients were then divided into two groups according to the year of BMT: up to or after 1990. The overall death rate dropped from 43 to 24%. Improvements are seen mostly for grafts from identical siblings (from 54 to 75%) and less for alternative donors (from 28 to 35%).[26]

Chronic GVHD is the major cause of death in long-term survivors of BMT. The relative risk of death is higher when BMT is performed more than 1 year after diagnosis, after acute GVHD or in the presence of active chronic GVHD 2 years after BMT. The actual survival rate is 85% in subjects without chronic GVHD and 69% in patients with GVHD. For this reason prophylaxis of GVHD is important: major changes are related to the increased use of cyclosporine with or without methotrexate.[27]

The continuing mortality after treatment, especially when only immunosuppressive therapy is given, is largely attribuible to a high risk of developing clonal marrow disorders (3.1% in BMT recipients versus 18.8% in patients managend only with immunosuppressive therapy). This risk is higher in subjects with congenital AA (elevated percentage of genetic abnormalities).

The risk of developing solid tumors is similar in patients treated with allogeneic BMT and in those treated with immunosuppressive therapy alone (2.2%).[27]

Allogeneic bone marrow transplantation in thalassemia major

BMT in thalassemia major was successfully accomplished in 1982. Much additional experience has been gained since. The improvements in results are related to better selection of the patients for BMT: patients with thalassemia major selected for allogeneic bone marrow transplant can be allocated to three classes with respect to the three following risk factors: entity of hepatomegaly, portal fibrosis on pre-transplant liver biopsy and chelating treatment before transplantion (Table 7).

Each patient with an HLA-identical donor is assigned to a protocol of treatment exclusively on the basis of the class: the first two classes are treated with the same protocol (cyclophosphamide, busulfan and cyclosporine) with a survival rate and EFS of 87% and 84%, respectively. Class 3 patients are prepared for BMT with the same drugs but the doses are lower because of the hepatic complication; the survival rate and EFS are 89% and 64% respectively.[28-30] Over 1,000 patients have now been transplanted in several highly experienced centers, the largest series being at Pesaro, Italy (Chief: G. Lucarelli). Table 8 summarizes the Pesaro experience of patient survival, disease-free survival and risk of GVHD in these three groups.[30]

Table 7. Classes of risk for patients with thalassemia major selected for allogeneic BMT.

Class 1: score 0	Iron chelation history: Score 0 = "regular", i.e. by subcutaneous infusion for 8-10 hours at least 5 days/week and started within 18 month from the 1st transfusion
Class 2: score 1-2	Score 1 = "irregular", i.e. any deviation from the above
Class 3: score 3	Hepatomegaly: Score 0 = liver less than 2 cm below the costal margin Score 1 = liver enlarged 2 cm or more Portal fibrosis on pre-transplant liver biopsy: Score 0 = no fibrosis Score 1 = presence of fibrosis

Table 8. The Pesaro experience.

Class	*N° of risk factors*	*Long-term survival*	*Disease-free survival (>10 yr)*	*Rejection*	*Mortality*
1	0	92%	85%	---	8%
2	1-2	84%	80%	5%	16%
3	3	61%	53%	16%	29%
3<17 yr	3	74%	46%	35%	24%
3>17 yr	3	63%	60%	4%	39%

The long-term effects of BMT in thalassemia are being monitored in patients transplanted between 1987-1995 and compared with those subjects matched for age and treated during the same period with conventional therapy. The incidences of fulminant sepsis and growth impairment are significantly higher in transplanted patients, whereas the occurrence of hypothyroidism, hypogonadism, and cardiomyopathy are higher in the other group of patients. Non-significant differences are observed in the incidences of diabetes, liver disease, and severe infections.[29-32]

After successful marrow transplantation, iron overload remains an important cause of morbidity in patients with thalassemia. Phlebotomy represents a safe, efficient and widely applicable method to decrease iron overload. One study demonstrates that moderately intensive phlebotomies can help to reduce iron overload and improve cardiac status. The same results can be obtained in the same time with desferroxamine.[33]

Allogeneic bone marrow transplantation in sickle cell disease

Hematopietic stem cells transplantations with bone marrow or umbilical cord blood from HLA-identical sibling donors have shown that sickle cell disease (SCD) can be cured with stabilization of prior organ damage.[34-36] In spite of this, very few patients with sickle cell anemia undergo an allograft procedure in comparison to β-thalassemic patients.This is probably due to the more variable clinical course of SCD and to the limitation of eligibility to those patients with advanced symptomatic disease (Table 9), often with neurologic and pulmonary vasculopathy.[34,35]

Although most children grafted with HLA-identical marrow survive free of SCD,[34-36] several follow-up evaluations of patients with stable engraftment are required to assess the effective cessation of clinical vaso-occlusive events, the beneficial effect of donor erythropoiesis on SCD-related organ damage and possible late effects of the transplants related to the administration of myeloablative chemotherapy.

Walters *et al.* studied 50 children transplanted with matched sibling marrow between 1991 and 1999: Kaplan-Meier probabilities of survival and event-free survival were, respectively, 94% and 84%. All patients were evaluated for late effects of BMT and for its impact on sickle-cell related central nervous system (CNS) and pulmonary disease. BMT established normal erythropiesis in most patients who had stable donor engraftment: complications related to SCD resolved, pulmonary function tests were stable and all patients with a prior history of stroke had stable or improved cerebral nuclear magnetic resonance imaging results. No patients had episodes of pain, stroke or acute chest syndrome. Linear growth improved.[34]

However, an analysis of outcome of patients with a high risk of stroke events, treated with allogeneic BMT versus periodic prophylactic blood transfusions did not demonstrate a significant difference in risk of CNS events between those two groups.[36]

Table 9. Inclusion criteria for transplantation in patients with SCD.

- Age of patient <16 years old
- HLA-identical sibling donor
- Stroke
- Recurrent acute chest syndrome
- Sickle cell nephropathy
- Bilateral proliferative retinopathy
- Osteonecrosis of multiple joints
- Chronic priapism
- Recurrent debilitating pain
- Chronic red cell transfusion requirements

References

1. Rivera GK, Pinkel D, Simone JV, et al. Treatment of acute lymphoblastic leukaemia: 30 years experience at S. Jude Children's Hospital. N Engl J Med 1993; 329:1289-95.
2. Pui Ch, Crist WM. Biology and treatment of acute lymphoblastic leukaemia. J Pediatr 1994; 124:491-503.
3. Ramsay NKC, Kersey JH. Indications for marrow transplantation in acute lymphoblastic leukaemia. Blood 1990; 75:815-8.
4. Reiter A, Schiappe M, Wolf DL, et al. Chemotherapy in 998 unselected childhood acute lymphoblastic leucemia patients. Results and conclusions of the multicenter trial ALL-BFM 86. Blood 1994; 84:3122-33.
5. Aricò M, Basso G, Conter V, et al. Good steroid response in vivo predicts a favourable outcome in children with T-cell acute lymphoblastic leucemia. Cancer 1995; 75:1684-93.
6. Chessels JM, Bailey C, Wheeler K, Richards SM. Bone marrow transplantation for high-risk childhood lymphoblastic leukaemia in first remission: experience in MRC UKALL X. Lancet 1992; 340:565-8.
7. Bordigoni P, Vernant JP, Sonillet G, et al. Allogeneic bone marrow transplantation for children with acute lymphoblastic leukaemia in first remission: a cooperative study of the Group d'Etude de la Greffe de Moelle Osseuse. J Clin Oncol 1989; 7:747-53.
8. Wells RJ, Matthews J, Plant G, et al. Treatment of newly diagnosed children and adolescents with AML: a report form the Children's Cancer Group. Blood 1996; 87:4979-89.
9. Michel G, Socie G, Gebhard F, et al. Late effects of BMT for children with AML in first complete remission: the impact of conditioning regimen without total body irradiation - a report from the Societé Française de Greffe de Moelle. J Clin Oncol 1997; 15:2238-46.
10. Liesner RJ, Leiper AD, Hann IM, Chessells JM. Late effects of intensive treatment for AML and MDS in childhood. J Clin Oncol 1994; 12:916-24.
11. Arcese W, Amadori S, Testi AM, et al. Allogeneic vs autologous BMT vs intensive chemotherapy in childhood AnLL during first complete remission: AIEOP experience. Bone Marrow Transplant 1991; 7:71-4.
12. Bacigalupo A, Vitale V, Corvo R. The combined effect of TBI and CyA on the risk of relapse in patients with acute myeloid leukemia undergoing allogeneic bone marrow transplantation. Br J Haematol 2000; 108: 99-104.
13. Kernan NA, Bartsch G, Ash RC, et al. Analysis of 462 transplantations from unrelated donors facilitated by the National Marrow Donor Program. N Engl J Med 1993; 328:593-602.
14. Clift RA, Storb R. Marrow transplantation for CML: the Seattle experience. Bone Marrow Transplant 1996; 17:S1-3.
15. Cambier N, Baruchel A, Schlageter MH, et al. Chronic myeloid leukemia: from biology to therapy. Hematol Cell Ther 1997; 39:41-8.
16. Novitzky N. Myelodysplastic Syndromes in children. A critical review of the clinical manifestations and management. Am J Hematol 2000; 63:212-22.
17. Aladjidi N, Casanova JL, Canioni D. Severe aplastic

anemia of neonatal onset: a single-center retrospective study of six children. J Pediatr 1998; 132:599-612.
18. Chan GC, Head DR, Wang WC. Refractory anemia with ringed sideroblasts in children: two disease with a similar phenotype? J Pediatr Hematol Oncol 1999; 21:418-23.
19. Emanuel PD. Myelodysplasia and myeloprolipherative disorders in chidhood: an update. Br J Haematol 1999; 105:852-63.
20. Bader Meunier B, Tchernia G, Mielot F, et al. Occurrence of myeloproliferative disorder in patients with Noonan syndrome. J Pediatr 1997; 130:885-9.
21. Runde V, de Witte T, Arnold R, et al. Bone marrow transplantation from HLA-identical siblings as first-line treatment in patients with MDS: early transplantation is associated with improved outcome. Chronic Leukemia Working Party of European Group for Blood and Marrow transplantation. Bone Marrow Transplant 1998; 21:255-61.
22. Hasle H, Arico M, Basso G, et al. Myelodysplastic syndrome, juvenile myelomonocytic leukaemia, and AML associated with complete or partial monosomy 7. European Working Group on MDS in childhood (EWOG-MDS). Leukemia 1999; 13:376-85.
23. Pitcher LA, Hann IM, Evans JP, Veys P, Chessells JM, Webb DK. Improved prognosis for acquired aplastic anemia. Arch Dis Child 1999; 80:158-62.
24. Ball SE. The modern management of SAA. Br J Haematol 2000; 110:41-53.
25. Heimpel H. Aplastic anemia before BMT and ATG. Acta Haematol 2000; 103:11-5.
26. Bacigalupo A, Oneto R, Bruno B, et al. Current results of bone marrow transplantation in patients with acquired severe aplastic anemia. Report of the European Group for Blood and Marrow transplantation. On behalf of the Working Party on Severe Aplastic Anemia of the European Group for Blood and Marrow Transplantation. Acta Haematol 2000; 103:19-25.
27. Gluckman E, Esperou-Bourdeau H, Baruchel A, et al. Multicenter randomized study comparing cyclosporin-A alone and ATG with prednisolone for treatment of severe aplastic anemia. Blood 1992; 79:2540-6.
28. Giardini C, Lucarelli G. Bone marrow transplantation for beta-thalassemia. Hematol Oncol Clin North Am 1999; 13:1059-64.
29. Piga A, Longo F, Voi V, Facello S, Miniero R, Dresow B. Late effects of bone marrow transplantation for thalassemia. Ann N Y Acad Sci 1998; 850:294-9.
30. Lucarelli G, Galimberti M, Giardini C, et al. Bone marrow transplantation in thalassemia. The experience of Pesaro. Ann N Y Acad Sci 1998; Jun 30; 850:270-5.
31. Gaziev D, Polchi P, Lucarelli G, et al. Second marrow transplants for graft failure in patients with Thalassemia. Bone Marrow Transplant 1999; 24:1299-306.
32. Li CK, Lai DH, Shing MM, Chik KW, Lee V, Yuen PM. Early iron reduction programme for Thalassemia patients after BMT. Bone Marrow Transplant 2000; 25:653-6.
33. Nietert PJ, Abboud MR, Silverstein MD, Jackson SM. Bone marrow transplantation versus periodic prophylactic blood transfusion in sickle cell patients at high risk of ischemic stroke: a decision analysis. Blood 2000; 15; 95:3057-64.
34. Walters MC, Storb R, Patience M, et al. Impact of BMT for symptomatic sickle cell disease: an interim report. Blood 2000; 95:1918-24.
35. Walters MC, Patience M, Leisenring W, et al. Barriers to bone marrow transplantation for sickle cell anemia. Biol Blood Marrow Transplant 1996; 2:100-4.
36. Bernaudin F. Results and current indications of bone marrow allograft in sickle cell disease. Pathol Biol 1999; 47:59-64.

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Indications and role of allogeneic bone marrow transplantation in childhood very high risk acute lymphoblastic leukemia in first complete remission

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ABSTRACT

Background and Objectives. **Acute lymphoblastic leukemia (ALL) accounts for approximately one third of all cancers in children and its outcome depends on risk factors at the time of diagnosis. While uniform chemotherapy adopted in multicenter studies provided a constant improvement in cure rates for standard risk patients, the results reached in very high risk patients have been disappointing. The objective of this review is to point out the role of allogeneic bone marrow transplantation (alloBMT) in very high risk childhood ALL on the basis of results from the current clinical trials.**

Evidence and information source. **Data covered by Medline and produced by the authors involved in ongoing international studies cover a vast "scenario" of children with very high risk ALL who underwent allogeneic BMT.**

State of art. **The author outlines the crucial point of very high risk factors in childhood ALL in order to identify those children who are at risk of early relapse. The main reasons for pursuing alloBMT in this particular category of patients concern poor prognostic factors such as molecular biology markers, structural chromosomal abnormalities and biological factors (poor prednisone response) including resistance to initial induction chemotherapy. AlloBMT in childhood ALL in first complete remission seemed to lead to a promising disease-free survival in this patient population when compared with chemotherapy. The principal biases of the retrospective studies were the variable very high risk eligibility criteria, the different first-line therapies adopted before alloBMT and above all the *waiting time to transplant* which could have accounted for some advantage to alloBMT patients versus chemotherapy patients.**

Perspectives. **The author touches upon the preliminary results of an ongoing international prospective study as an example of reaching a consensus in the controversial treatment of childhood very high risk ALL. This attempt should provide more information regarding the role of alloBMT in this setting and should cover an area of particular interest.**

Key words: ABMT, acute lymphoblastic leukemia

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In the last decade many data have emerged from single institute or multicenter trials involving children affected by very high risk ALL in 1st complete remission (CR), but variable results have been shown regarding the event-free survival (EFS) which ranges from 50% to 70% in patients treated with alloBMT[1-6] and from 10 to 40% in those treated with chemotherapy.[6-11] These data primarily depend on the selection of the patients on the basis of very high risk eligibility criteria, the intensity of the first-line treatment and subsequently the quality of the bone marrow remission.

Current definition of very high risk factors in childhood ALL

One of the crucial points explaining different results obtained by different strategies is the definition of the very high risk criteria over the years. International co-operative groups (POG, CCG, I-BFM-SG, AIEOP) are using a similar risk classification scheme including the following very high risk criteria in the ongoing trials: a) genetic features (MLL rearrangements and BCR ABL fusion which affect less than 10 % of children with ALL), b) hyperleukocytosis (white blood cell count $\geq$ 100,000/mm^3) and/or T-immunophenotype combined with *poor* prednisone response, c) induction failure and, more recently, d) minimal residual disease (MRD) persistence. Other previous risk factors such as myeloid markers, *poor* prednisone response *per se*, hyperleukocytosis or T-immunophenotype alone, are no longer considered poor prognosis indicators.[11-15]

On the other hand international studies recently demonstrated that TEL-AML 1 expression in leukemic blast cells at diagnosis is a powerful favorable prognostic factor that provides clinically useful information independent of age, leukocyte count and other parameters at the onset of the leukemia.[16-18]

In synthesis clinical, biological, genetic and immunologic features can serve as useful markers for formulating a prognosis for very high risk ALL patients and must be taken into consideration when the clinician plans to apply a treatment.

Value of transplantation strategy for very high risk childhood ALL in 1st CR

The role of allogeneic hematopoietic stem cell transplantation in the treatment of very high risk ALL in 1st CR is still debatable as very few studies have so far tried to establish a comparative evaluation of alloBMT versus chemotherapy.

While single center studies, such as those of Brochstein[1] and Sanders[2] and the multicenter trial of Bordigoni[3] showed promising results for alloBMT patients, in 1992 Chessels,[4] on behalf of the MRC, did not find a statistically significant difference between prospectively studied patients managed with alloBMT or chemotherapy for high risk childhood ALL in 1st CR. In 1996 Saarinen demonstrated, in a NOPMO case-control study (22 alloBMT patients versus 44 matched controls), a superior outcome for the alloBMT procedure (10 year DFS of 73% vs 50%): however the eligibility criteria of this study selected a group of patients without very high risk features at diagnosis.

An Italian co-operative study,[5] based on homogeneous very high risk criteria (including most of the adverse prognostic factors most recently employed by international study groups) and using a matched design to control for both known very high risk prognostic factors and the time-to-transplant bias, showed no significant statistical difference between the two groups (4-year DFS of 58.5% and 47.7% for alloBMT vs. chemotherapy patients, respectively). Despite this, the alloBMT curve remained stable reaching a plateau after one year whereas the chemo therapy curve was more likely to fall since patients continued to experience relapse after 4 years.

Ongoing international prospective study for very high risk childhood ALL in 1st remission

Recently The EBMT and I-BFM-SG set up a prospective randomized (by genetic chance) trial with the aim of comparing the role of alloBMT vs. chemotherapy in the treatment of very high risk childhood ALL. The homogeneous criteria adopted in defining very high risk patients were as follows: cytogenetic abnormalities such as t(9;22) or t(4;11), poor prednisone response and T-ALL or WBC more than 100,000/mm^3, induction failure. Patients having a suitable HLA A, B, DR sibling donor underwent alloBMT as soon as they obtained the 1st CR.

At present the main goal of this ongoing cooperative study is to recruit a sufficient number of patients (72 alloBMT and 238 chemotherapy patients, respectively) in order to perform an intention-to-treat analysis which will be definitive in 1992. With an actual median follow–up of 27 months the z-year DFS by treatment performed and adjusted by waiting time to transplant is 58.4% (8.1 s.e.) and 53.4% (3.8 s.e.) for alloBMT and chemotherapy patients, respectively. In this interim analysis one should take into account not only the short follow-up but also some deviations from the study in that some patients received a match/mismatch unrelated donor BMT(MUD BMT) or alternative donor BMT (data not published). From this study and on the basis of some recent results of MUD BMT in childhood ALL in 1st CR[19-23] one can foresee a trend in favor of this still debatable procedure at least for those particular subgroups of very high risk patients who lack a suitable sibling donor.

As a matter of fact the effectiveness of chemotherapy in patients with adverse genetic features, such as MLL gene arrangements or BCR ABL fusion, especially in association with a poor prednisone response and/or a high WBC count at diagnosis (more than 100,000/mm^3), is dismal because these patients have a probability of EFS of no more than 20% in the long-term.[22] Patients who fail induction therapy could constitute another subgroup of patients for whom even an experimental type of BMT such as haplo BMT should be offered without delay when an HLA identical sibling or an unrelated donor is not available.

Last but not least, persistent MRD within two to three months after the diagnosis of very high risk ALL has been identified as another bad prognosis factor so that it constitutes a new indication for an alloBMT.

Conclusions

In view of the more recent valid therapeutic options to offer to children with very high risk ALL, it is mandatory to follow the continuous progress both of alloBMT and chemotherapy reached by intergroup co-operative studies. The future challenge regards decreasing transplant-related mortality by providing better treatment support so that a better overall survival can be

achieved. However the relapse rate which, in the past, constituted one of the major barriers to curing childhood very high risk ALL should be overcome by new prospective clinical trials chemotherapy or alloBMT.

References

1. Brochstein JA, Kernan NA, Groshen S, et al. Allogeneic bone marrow transplantation after hyperfractionated total body irradiation and Cyclophosphamide in children with acute leukemia. N Engl J Med 1987; 317: 1618-24.
2. Sanders JE, Thomas ED, Buckner CD, et al. Marrow transplantation for chldren with acute lymphoblastic leukemia in second remission. Blood 1987; 79:517-26.
3. Bordigoni P, Vernant JP, Souillet G, et al. Allogeneic bone marrow transplantation for children with acute lymphoblastic leukemia in first remission: a cooperative study of the Group d'Etude de la Greffe de Moelle Osseuse. J Clin Oncol 1989; 7:747-53.
4. Chessels JM, Bailey, Wheeler K, et al. Bone marrow transplantation for high risk childhood lymphoblastic leukemia in first remission: experience of MRC UK ALL X. Lancet 1992; 340:565-8.
5. Uderzo C, Valsecchi MG, Balduzzi A, et al. Allogeneic bone marrow transplantation versus chemotherapy in high risk childhood acute lymphoblastic leukemia in first remission . Br J Haematol 1997; 96:387-94.
6. Saarinen UM, Mellander L, Nyson K, et al. Allogeneic bone marrow transplantation in first remission for children with very high risk acute lymphoblastic leukemia: a retrospective case control-study in Nordic Countries. (NOPHO) Bone Marrow Transplant 1996; 17:357-63.
7. Gaynon PS, Bleyer WA, Steinherz PG, et al. Day 7 marrow response and outcome for children with ALL and unfavourable presenting features. Med Ped Oncol 1990; 27:273-9.
8. Rivera GK, Pinkel D, Simone JV, et al. Treatment of ALL: 30 years' experience at St. Jude Children's Research Hospital. N Engl J Med 1993; 329:1289-95.
9. Conter V, Rizzari C, Aricò M, et al. Preliminary results of the AIEOP '91 study for childhood ALL. Med Ped Oncol 1995; 17:283-9.
10. Reiter A, Schrappe M, Wolf Dieter L, et al. Chemotherapy in 998 unselected childhood ALL patients: results and conclusions of the multicenter trioal ALL BFM 86. Blood 1994; 84:3122-33.
11. Pui CH, Evans WE. Acute Lymphoblastic Leukaemia. New Engl J Med 1998; 333:605-12.
12. Behn FG, Raimondi SC, Frested JL, et al. Rearrangement of the MLL gene confers a poor-prognosis in childhood ALL,regardless of presenting age. Blood 1996; 87:2870-7.
13. Schlieben S, Borkhardt A, Reinisch I, et al. Incidence and clinical outcome of children with BCR/ABL positive ALL. A prospective RT-PCR study based on 673 patients enrolled in the German pediatric multicenter therapy trials ALL BFM-90 and CoALL-05-92. Leukemia 1996; 10:957-63.
14. Childhood ALL collaborative Group: duration of maintenance chemotherapy in ALL: overview of 42 trials involving 12.000 randomized children. Lancet 1996; 347:1783-8.
15. Steinherz PG, Gaynon PS, Breneman JC, et al. Cytoreduction and prognosis in ALL. The importance of early marrow response: report from the Childrens Cancer Group. J Clin Oncol 1996; 14:389-98.
16. Pui CH, Schell MJ, Raimondi SC, et al. Myeloid-antigen expression in childhood acute lymphoblastic leukemia. N Engl J Med 1991; 325:1378-9.
17. Mc Lean TW, Ringold S, Neuberg D et al: TEL/AML 1 dimerizes and is associated with a favorable outcome in childhood ALL. Blood 1996; 88:4252-8.
18. Liang DC, Chow TB, Chen JS, et al. High incidence of TEL/AML 1 fusion resulting from a criptic t (12; 21) in childhood B-lineage ALL in Taiwan. Leukemia 1996; 10:991-3.
19. Balduzzi A, Gooley T, Anasetti C, et al. Unrelated donor marrow transplantation in children. Blood 1995; 86:3247-55.
20. Davis S, Wagner JE, Shu Xo, et al. Unrelated donor marrow transplantation for children with acute leukemia. J Clin Oncol 1997; 15:557-65.
21. Green A, Clarke E, Hunt L, et al. Children with ALL who receive T-cell-depleted HLA mismatched marrow allografts from unrelated donors, have an increase incidence of primary graft failure but a similar overall transplant outcome. Blood 1999; 94:2236-46.
22. Biondi A, Cimino G, Pieters R, Pui CH. Biological and therapeutic aspects of infant leukemia. Blood 2000; 96:24-33.
23. Sierra J, Radich J, Hansen JA, et al. Marrow transplants from unrelated donors for treatment of Philadelphia chromosome positive acute lymphoblastic leukemia. Blood 1997; 90:1410-4.

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Non-myeloablative allogeneic stem cell transplantation in children

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Allogeneic stem cell transplantation (alloSCT) is a well documented treatment for a variety of malignant and non-malignant diseases both in adults and pediatric patients, nevertheless knowledge about its consequences is still incomplete.

It is a matter of fact that transplant-related-toxicity still represents a major clinical problem. Although there is a wide variation between reports, due largely to differences in the patient populations studied, early mortality incidence is estimated to be 10-30%. The results in children are consistently better than those in adults, this being mainly due to the lower incidence and severity of organ toxicity and graft-versus-host-disease (GVHD) in younger patients.[1-3]

The probability of post-transplant short-term fatal outcome is particularly relevant because many children are transplanted at a stage of disease at which the average survival expectancy without transplant may be considerably longer. This is verified both for acute leukemia in first remission and non-neoplastic diseases, such as thalassemia and metabolic diseases. Furthermore growing children are particularly vulnerable to delayed adverse sequelae and since transplanted children are surviving longer, late effects are becoming more apparent. Conditioning regimens and the transplant process itself account for a variety of late effects observed in childhood. Thyroid abnormalities, gonadal failure, growth hormone deficiency are frequently reported, particularly in children receiving total body irradiation (TBI)-containing regimens. Treatment-related neurologic complications are also observed, mostly in patients undergoing transplant during infancy. Chronic pulmonary dysfunction, particularly restrictive defects, as well as ophthalmologic, dental, cardiac and bone complications are well documented. Furthermore the immune system may be impaired for many years after a transplant. The risk of secondary malignant diseases, especially for patients prepared with TBI-containing regimens is particularly relevant.[1,2,4]

Finally, it is now clear that the space making property of myeloablation may damage the marrow stromal microenvironment and in particular its fibroblast component, affecting its intrinsic ability to support hematopoiesis. Besides it was found that the number of early hematopoietic progenitors is reduced after a transplant, suggesting a permanent reduction in the stem cell reservoir.[5]

Attempts to develop less toxic procedures making marrow transplant safer and more generally applicable in a pediatric population are indeed very attractive.

Myeloablative doses of chemotherapy, with or without TBI, are routinely used as conditioning regimens, based on the concept that high doses are necessary to open space for donor stem cell engraftment and to suppress host immunity in order to prevent rejection.[6-8] Although this principle was a long and strongly held dogma, recent experimental evidence supports the hypothesis that engraftment itself can create space through a subclinical GVHD effect and that donor T-cells help to eliminate or to inactivate residual host T-lymphocytes which would otherwise mediate rejection.[9-11] Furthermore, according to several reports the onset of mixed hematopoietic-chimerism is likely to ensure a successful transplant both in malignancies and in non-neoplastic diseases such as thalassemia and immunodeficiencies.[12-15]

In malignant diseases the preparative regimen is also deemed essential for eliminating or at least reducing residual leukemic clones.[7,8,16] Although is well documented that the resistance to intensive doses of therapy by residual tumor cells accounts for disease recurrence, attempts to improve disease-free-survival by means of conditioning intensification are associated with an increase of morbidity and transplant-related-mortality (TRM). The better results achieved in the last ten years are explained by the improvement of supportive care rather than by higher effectiveness against the underlying dis-

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ease. This observation is confirmed by a constant post-transplant rate of relapse through the years.

Moreover it has became increasingly evident that an important component of the curative potential of allogeneic stem cell transplantation may be achieved through the induction of a state of host-versus-graft tolerance, giving donor-derived T-lymphocytes the opportunity to recognize and eradicate tumor cells and host abnormal as well as normal stem cells.

Data are accumulating showing that the therapeutic benefit of allotransplants in preventing relapse is largely explained by a donor T-lymphocyte mediated immunologic effect (graft versus leukemia = GVL) rather than by physical elimination of tumor cells by high doses of cytoreductive therapy. Eradication of the host immuno-hematopoietic system by means of adoptive allogeneic cell therapy was confirmed in pre-clinical and clinical studies. This observation is well-documented in patients with chronic myeloid leukemia (CML) who gave relapsed after transplant but who may achieve complete cytogenetic and molecular remission following donor lymphocyte infusion (DLI). Moreover acute myeloid leukemia (AML), acute lymphoid leukemia (ALL) and others malignancies such as non-Hodgkin's lymphoma (NHL), chronic lymphatic leukemia (CLL), melanoma and some solid tumors seem to be susceptible to a graft-versus-tumor effect (GVT), although clinical data are less impressive. Furthermore, the effectiveness of DLI therapy sustains the hypothesis that long-term disease control may be obtained without myeloablative conditioning.[9,10,17,18]

Despite progress in supportive therapy, myeloablative regimen toxicity still leads to a high incidence of acute side affects and contributes, in combination with GVHD, to considerable TRM. These complications occur more frequently in adults or in the unrelated setting transplants, especially from unrelated donors, are therefore still performed mainly in young patients with recurrence of leukemia, as the risk of severe complications and mortality is too high for routine application in non-malignant but potentially curable diseases, such as hemoglobinopathies, metabolic diseases, immunodeficiencies or acquired aplastic anemia.

Clinical studies have recently shown that allogeneic engraftment can be accomplished through a different strategy without myeloablative doses of chemotherapy. The possibility of achieving stable engraftment without myeloablation and of completely eradicating leukemia by adoptive allogeneic cells suggested the working hypothesis for developing a new approach for allogeneic bone marrow transplantation. This hypothesis is based on the possibility of using donor T-cells to eradicate both host malignant and non-malignant clones avoiding the need of complete myeloablation. This strategy is primarily designed to facilitate donor cell engraftment (by achieving tolerance toward donor cells) and less to eradicate the underlying disease by means of conditioning.[9,10,19-36] The reduction of acute and chronic complications related to intensive therapy toxicity would improve the outcome of the procedure, extending its feasibility to patients otherwise deemed ineligible according to standard criteria.

The development of less toxic and non-myeloablative conditioning regimens was first investigated at the Hadassah University in Jerusalem and at the MD Anderson Institute in Houston which have pioneered the new concept of non-myeloablative allograft (or miniallograft as termed by some authors to highlight the low-intensity of the protocol). Subsequently other Institutions world-wide started pilot experiences and an increased amount of data is now available.[9,10,19-42]

The intensity of the proposed regimens may differ in terms of the real capacity of hematopoietic recovery without stem cell support. Indeed most of the currently used protocols have never been tested in clinical setting without stem cell support; they are of lower in intensity than conventional schedules but variable degrees of hematopoietic recovery may result according to the hematopoietic reserve of each patient. This difference in intensity should be included in the analysis of the results reported by different institutions about the procedure toxicity.

If the underlying concept of this transplant is that its curative activity is mediated by an immunologic reaction rather than by chemical eradication of the disease, the importance of tumor mass at the time of transplant still remains a matter of debate. Some authors suggest that even refractory or progressive diseases may benefit from allogeneic immunotherapy and emphasize that excessive pre-transplant cytoreduction may lead to normal host hematopoietic environment damage. Others remark that allogeneic immunotherapy can be promising only at the stage of minimal residual disease.[10,11,17,20,25,27-29,32,40]

Many non-ablative conditioning regimens have been tested by various teams or by the same team in different settings of patients. To date it seems that fludarabine or low-doses of TBI play a crucial role in these less intensive programs providing enough immunosuppression to allow allogeneic engraftment without severe myelotoxicity.[25,27,28]

In most of these settings, allotransplant may be considered the platform for subsequent adoptive immunotherapy using donor lymphocytes. In

fact the common feature of these protocols is that intensive short-term immunosuppression is performed in order to induce host tolerance against donor cells which in turn enable a potent GVL effect by large numbers of donor-derived immunocompetent T-cells. GVL is then enhanced by subsequent DLI, if needed.

Here below we report the conditioning regimens adopted by most Institutions.

Condition regimens

Hadassah University Hospital (Jerusalem). The basic protocol is fludarabine (30 mg/m^2/d for 6 consecutive days), oral busulfan 4 mg/kg/d for 2 consecutive days and anti-T-lymphocyte globulin ATG (5-10 mg/kg/d Fresenius for 4 consecutive days). This schedule has been subsequently modified in selected patients with genetic disease or aplastic anemia in that busulfan was substituted by cyclophosphamide, or low dose TBI in single dose (200 cGy).

MD Anderson (Houston). Fludarabine 30 mg/m^2/d and Ara-C 2 g/m^2/d for 4 days, idarubicin 12 mg/m^2/d for 3 days. Patients previously exposed to fludarabine received Ara-C 1 g/m^2/d and 2-CDA at a dose of 12 mg/m^2/d for 5 days.

Fred Hutchinson (Seattle). Low-dose TBI (200 cGy) in a single dose.

Ospedale San Martino (Genoa). Thiothepa 10 mg/kg 1 dose, then cyclophosphamide 50 mg/kg/d for 2 days.

Another approach based on autografting followed by non-myeloablative allograft was proposed in alternative to this protocol. In this case conditioning includes fludarabine 30 mg/kg/d and cyclophosphamide 300 mg/m^2/d for 3 days.

San Raffaele Hospital (Milan). Thiothepa 5 to 15 mg/kg according to the age of the patient, fludarabine 60 mg/m^2 and cyclophosphamide 60 mg/kg. Infusion of engineered lymphocytes (thymidine kinase gene) in subsequent weeks was planned.

Harvard Medical School (Boston). Cyclophosphamide 50 mg/kg/d for 4 days and thymic irradiation.

University Hospital Carl Gustav (Dresden). Busulfan 3.3 mg/kg/d i.v. or 4 mg/kg/d p.o. for 2 days and fludarabine 30 mg/m^2/d for 5 days.

In order to infuse a higher number of CD34$^+$ cells and T-lymphocytes, peripheral blood stem cells were used in most cases. In some cases bone harvest and in a few patients cord blood were used as the stem cell source. GVHD prophylaxis consisted of cyclosporinA (CSA), CSA and a short course of methotrexate (MTX), CSA and methylprednisolone, CSA and mofetil mycophenolate (MMF). CSA administration was planned for a shorter period of time compared to standard protocols and then modulated according to GVHD severity.

In most cases the donors were matched siblings, but cases of unrelated donors have been described. Some cases of 1-3 locus mis-matched transplants are reported.

These heterogeneous conditioning regimens were employed in different settings of patients. In the first experiences these protocols were suggested for patients aged more than 50-55 years and therefore not eligible for conventional transplant, or for heavily pre-treated younger patients with reduced performance status or who had relapsed after a first autologous or allogeneic transplant.[10,19-20,21,27-29] Conversely the Hadassah team explored these transplants also in patients eligible for standard treatment and in non-malignant diseases.[10,32-34,37,40,41] These wide differences make the comparison of data and results from various reports difficult; furthermore it must be remembered that these results emerge from pilot and ongoing studies with a short follow-up.

Results and Conclusions

Based on cumulative experience reported in the literature it may be argued that allogeneic non-myeloablative regimens are much better tolerated than standard protocols, leading to a similar or even lower toxicity than conventional chemotherapy, improving quality of life and cost effectiveness.[25,27,28]

Limited non-hematologic and hematologic toxicity and faster recovery compared to those produced by standard protocols were observed in almost all cases. Similarly, transfusional support and antibiotic administration as well as time spent in hospital were reduced in the majority of recipients of non-myeloablative regimens compared to in recipients of standard transplants. Engraftment and stable full or mixed chimerism were observed in about 90% of patients receiving either HLA-identical or mis-matched transplants, but this percentage reached 100% in some studies. Interestingly it has been well documented that host-derived cells may slowly disappear leading to complete chimerism development. This confirms that once a state of tolerance is achieved, donor cells engraftment is progressively able to replace normal and malignant cells through a non-clinical GVH reaction. Lasting mixed chimerism was observed also in haplo-identical settings.[10,12,13,27,28]

Concerning the control of underlying disease, results should be carefully reviewed in consideration of the advanced stage of disease in most patients, the heterogeneity of the cases and the relatively short follow-up. Preliminary results confirm that non-ablative regimens may control the neoplastic disease also in advanced stage, but, albeit encouraging results, recurrence of disease remains the most important cause of trans-

plant failure. The role of DLI is not completely clarified; nonetheless it seems of crucial importance in leading to long-term disease-free-survival in some conditions.[10,11,17,19-21,23,25,27,28,31,32,36]

As far as the overall incidence of acute GVHD and its severity is concerned, although conclusive data are not available yet, there appears to be no significant differences between the non-myeloablative regimens and standard procedures. HLA disparity seems to be the most important factor. In the majority of cases severe acute GVHD appeared following immunosuppression withdrawal or DLI. Nevertheless all cases were responsive to treatment. It is notable that acute GVHD onset is generally delayed and often occurs after full recovery from conditioning toxicity; this definitely contributes to mitigating the severity of GVHD-related tissue damage.[10,27,28,32]

The reduced organ and hematologic toxicity and the delayed onset of account GVHD mainly account for the very low incidence of TRM; based on published reports only a few patients died as a consequence of procedure-related toxicity within the first 100 days post-transplant.[25,27,28,31]

The incidence and severity of chronic GVHD is still difficult to assess because of the short period of observation, but preliminary data do not seem differ from those found with standard protocols.[25,27,28,31]

The results in patients with non-malignant disease are noteworthy. Nearly all cases (thalassemia major, severe aplastic anemia, Fanconi's anemia, osteopetrosis, severe immunodeficiency, chronic granulomatous disease, Blackfan-Diamond syndrome and Gaucher's disease) were transplanted at Hadassah University. The procedure was well tolerated in all patients with some of them going through the whole procedure without blood product support or septic episodes. All patients obtained complete donor cell engraftment, occasionally after a transient stage of mixed-chimerism. Only two patients with advanced thalassemia showed autologous reconstitution following transient mixed-chimerism. The experience of Hadassah University in this setting of patients should be considered very encouraging.[10, 32,33,34,37,38,41]

In the last years the feasibility of self-reactive lymphocyte elimination by myeloablative conditioning regimens was documented, suggesting the existence of graft-versus-autoimmunity mediated by donor lymphocytes. An international committee of rheumathologists and transplant experts suggested autologous or allogeneic transplant to cure life-threatening autoimmune diseases, despite concern about the toxicity of myeloablation.[43] Recently the elimination of self-reactive clones was demonstrated following a non-myeloablative transplant. If these preliminary data are confirmed in other subjects, allogeneic non-ablative regimens might be suggested in this setting also for children affected by life-threatening autoimmune diseases.

In conclusion, it is well demonstrated that non-myeloablative regimens allow the extension of transplant to patients otherwise not eligible according to standard criteria, and this represents a very important clinical result.

To date GVHD and disease recurrence represent the issues that on which investigations should be concentrated, particularly concerning the real capacity of this approach to eradicate the neoplastic clones of the recipient.

The role of this innovative procedure in patients eligible for standard protocols will be a matter of debate in the coming years. Due to the relatively short period of observation, the small cohort of patients and the heterogeneity of both patients and underlying diseases, data must be carefully evaluated.

Furthermore as children have a long life expectancy they have a higher chance of developing secondary malignancies; less intensive conditioning regimens not involving TBI (or involving low dose TBI), lead to less DNA damage and chromosomal breakage, certainly lowering this risk.

At present, many patients with non-malignant diseases, potentially curable with transplant, can not be submitted to this definitive therapy because of the high TRM. For these subjects, most of them in pediatric age, non-myeloablative transplants may represent a promising therapeutic possibility. If the results are confirmed in this setting, stem cell transplantation will probably represent a real option for many patients affected by heterogeneous incurable diseases who are currently precluded from this procedure in consideration of the high risk of death or severe chronic complications related to myeloablation.

Finally this strategy may be considered safe enough to be carried out in some circumstances in an outstanding setting, with evident reduction of transplant-related costs.

Larger numbers of patients and longer periods of observation are required to assess whether non-myeloablative regimens result in better outcomes than those achieved by conventional myeloablative transplant. Randomized studies comparing non-myeloablative transplant to myeloablative ones should therefore be planned in the near future.

References

1. Kinsey SE. Pediatric problems. In: Barret J. and Treleaven J. Eds. The clinical practice of stem cell transplantation, Oxford: Isis Medical Media; 1998. pp.839-48.
2. Pession A, Rondelli R, Paolucci P, et al. Hematopoietic stem cell transplantation in childhood: report from the bone marrow transplantation group of the Associazione Italiana Ematologia Oncologia Pediatrica. Haematologica 2000; 85:638-46.
3. Garaventa A, Porta F, Rondelli R, et al. Early deaths in children after BMT. Bone Marrow Transplantation Group of the Italian Association for Pediatric Hematology and Oncology (AIEOP) and Gruppo Italiano Trapianto di Midollo (GITMO). Bone Marrow Transplant 1992; 10:419-23.
4. Sanders JE. Growth and development after hematopoietic cell transplantation. In: Thomas ED, Blume KG, Forman SJ Eds. Hematopoietic cell transplantation. Malden: Blackwell Science; 1999. pp. 764-75.
5. Galotto M, Berisso G, Delfino L, et al. Stromal damage as consequence of high-dose chemo/radiotherapy in bone marrow transplant recipients. Exp Hematol 1999; 27:1460-6.
6. Tutschka PJ. Marrow ablation: the need for space, immune suppression, and malignant cell eradication. In: Atkinson K. Eds. Clinical bone marrow and blood stem cell transplantation. Cambridge: Cambridge University Press; 2000 pp. 13-8.
7. Buckner CD, Besinger WI. Preparative Regimens.. In: Thomas ED, Blume KG, Forman SJ eds. Hematopoietic cell transplantation. Malden: Blackwell Science; 1999 pp. 123-34.
8. Phillips GL. Conditioning regimens. In: Deeg HJ, Klingemann HG, Phillips GL, van Zant G. eds. A guide to blood and marrow transplantation. Berlin: Springer; 1999. pp. 53-67.
9. Slavin S, Nagler A, Naparstek E, et al. Immunotherapy of leukemia in conjunction with non-myeloablative conditioning: engraftment of blood stem cells and eradication of host leukemia with non-myeloablative conditioning based on Fludarabine and anti-thymocyte globulin (ATG). Blood 1996; 88:614a.
10. Slavin S, Nagler A, Naparstek E, et al. Nonmyeloablative stem cell transplantation and cell therapy as an alternative to conventional bone marrow transplantation with lethal cytoreduction for the treatment of malignant and nonmalignant hematologic diseases. Blood 1998; 91:756-63.
11. Storb R. Nonmyeloablative preparative regimens: experimental data and clinical practice. In: Perry MC editor. American Society of Clinical Oncology Educational Book. Alexandria, VA: American Society of Clinical Oncology; 1999 pp.241-5.
12. Storb R, Yu C, McSweeney P. Mixed chimerism after transplantation of allogeneic hematopoietic cells. In: Thomas ED, Blume KG, Forman SJ eds. Hematopoietic cell transplantation. Malden: Blackwell Science; 1999 pp. 287-95.
13. Kapelushnik J, Or R, Aker M, et al. Allogeneic cell therapy of severe beta thalassemia major by displacement of host stem cells in mixed chimera by donor blood lymphocytes. Bone Marrow Transplant 1997; 19:96-8.
14. O'Reilly RJ, Friedrich W, Small TN Hematopoietic cells transplantation for immunodeficiency diseases. In: Thomas ED, Blume KG, Forman SJ eds. Hematopoietic cell transplantation. Malden: Blackwell Science; 1999 pp.1154-72.
15. Lucarelli G, Clift RA. Marrow transplantation in Thalassemia. In: Thomas ED, Blume KG, Forman SJ eds. Hematopoietic cell transplantation. Malden: Blackwell Science; 1999 pp. 1137-44.
16. Deeg HJ. Rationale and indications for transplantation. In: Deeg HJ, Klingemann HG, Phillips GL, van Zant G. eds. A guide to blood and marrow transplantation. Berlin: Springer; 1999 pp. 7-14.
17. Kolb HJ. Management of relapse after hematopoietic cell transplantation. In: Thomas ED, Blume KG, Forman SJ, eds. Hematopoietic cell transplantation. Malden: Blackwell Science; 1999 pp. 929-36.
18. Collins RH Jr, Shpilberg O, Drobyski WR, et al. Donor leukocytes infusion in 140 patients with relapsed malignancy after allogeneic bone marrow transplantation. J Clin Oncol 1997; 15:433-44.
19. Giralt S, Estey E, Albitar M, et al. Engraftment of allogeneic hematopoietic progenitor cells with purine analog-containing chemotherapy: harnessing graft-versus-leukemia without myeloablative therapy. Blood 1997; 89:4531-6.
20. Khouri IF, Keating M, Körbling , et al. Transplant-lite: induction of Graft-Versus-Malignancy using fludarabine-based nonablative chemotherapy and allogeneic blood progenitor-cell transplantation as treatment for lymphoid malignancies. J Clin Oncol 1998; 16: 2817-24.
21. Carella AM, Lerma E, Dejana A, et al. Engraftment of HLA-matched sibling hematopoietic stem cells after immunosuppressive conditioning regimen in patients with hematologic neoplasias. Haematologica 1998; 83:904-9.
22. McSweeney PA, Wagner JL, Maloney DG, et al. Outpatient PBSC allografts using immunosuppression with low-dose TBI before, and cyclosporine (CSP) and mycophenolate mofetil (MMF) after transplant. Blood 1998; 92:519a (Abstr. 2133).
23. Sykes M, Preffer F, McAfee S, et al. Mixed lymphohematopoietic chimerism and graft versus lymphoma effect are achievable in adult recipients following nonmyeloablative therapy and HLA-mismatched donor bone marrow transplantation. Lancet 1999; 353: 1755-9.
24. Childs R, Clave E, Contentin N, et al. Engraftment kinetics after nonmyeloablative allogeneic peripheral blood stem cell transplantation: full donor T-cell chimerism precedes alloimmune responses. Blood 1999; 94:3234-41.
25. Bacigalupo A. Hematopoietic stem cell transplant after reduced intensity conditioning regimen (RI-HSCT): report of a workshop of the European Group for Blood and Marrow Transplantation (EBMT). Bone Marrow Transplant 2000; 25:803-5.
26. Bornhäuser M, Thiede C, Schuler U, et al. Dose-reduced conditioning for allogeneic blood stem cell transplantation: durable engraftment without antithymocyte globulin. Bone Marrow Transplant 2000; 26:119-25.
27. Carella AM, Champlin R, Slavin S, et al. Mini-allografts: ongoing trials in humans. Bone Marrow Transplant 2000; 25:345-50.
28. Carella AM, Giralt S, Slavin S. Low intensity regimens with allogeneic hematopoietic stem cell transplantation as treatment of hematologic neoplasia. Haematologica 2000; 85:304-13.
29. Grigg A, Bardy P, Byron K, et al. Fludarabine-based non-myeloablative chemotherapy followed by infusion of HLA-identical stem cells for relapsed leukaemia and lymphoma. Bone Marrow Transplant 1999; 23:107-10.
30. Niederwiesar D, Wolff D, Mantovani L, et al. Hematopoietic stem cell transplant (HSCT) from HLA matched and mismatched unrelated donors using

non-myeloablative regimen. Blood 1999; 94:581a.
31. Martino R, Sierra J. Allogeneic hematopoietic stem cell transplantation after immunosuppressive but non-myeloablative conditioning "miniallografts" are no small matter. Haematologica 1998; 83:865-7.
32. Nagler A, Slavin S, Varadi G, et al. Allogeneic peripheral blood stem cell transplantation using a fludarabine-based low intensity conditioning regimen for malignant lymphoma. Bone Marrow Transplant 2000; 25:1021-8.
33. Nagler A, Or R, Naparstek E, et al. Matched unrelated bone marrow transplantation using a non-myeloablative conditioning regimen. Blood 1998; 92: 289a.
34. Nagler A, Ackerstein A, Kapelushnik J, et al. Donor lymphocyte infusion post-non-myeloablative allogeneic peripheral blood stem cell transplantation for chronic granulomatous disease. Bone Marrow Transplant 1999; 24:339-42.
35. Rao AS. Microchimerism and its role in the induction of transplantation tolerance. Cancer Res Ther Control 1998; 7:7-11.
36. Spitzer TR, McAfee S, Sackstein S, et al. Intentional induction of mixed chimerism and achievement of antitumor responses after nonmyeloablative conditioning therapy and HLA-matched donor bone marrow transplantation for refractory hematologic malignancies. Biol Blood Marrow Transplant 2000; 6:309-20.
37. Kapelushnik J, Or R, Slavin S, Nagler A. A fludarabine-based protocol for bone marrow transplantation in Fanconi's anemia. Bone Marrow Transplant 1997; 20:1109-10.
38. Or R, Kapelushnik J, Naparstek E, et al. Second transplantation using allogeneic peripheral blood stem cells in a b-thalassemia major patient featuring stable mixed chimerism. Br J Haematol 1996; 94:285-7.
39. Aker M, Varadi G, Slavin S, et al. Fludarabine-based protocol for human umbilical cord blood transplantation in children with Fanconi's anemia. J Pediatr Hematol Oncol 1999; 21:237-9.
40. Nagler A, Or R, Naparstek E, Varadi G, Slavin S. Second allogeneic stem cell transplantation using non-myeloablative conditioning for patients who relapsed or developed secondary malignancies following autologous transplantation. Exp Hematol 2000; 28:1096-104.
41. Kapelushnik J, Mandel H, Varadi G, et al. Fludarabine-based protocol for haploidentical peripheral blood stem cell transplantation in Hurler Syndrome. J Pediat Hematol Oncol (submitted).
42. Corradini P, Bregni M, Tarella C, et al. Allografting for high-risk hematological and solid tumors: reduced intensity chemotherapy followed by reinfusion of lymphocytes engineered with thymidine kinase gene. Bone Marrow Transplant 2000; 25:396a.
43. Slavin S, Nagler A, Varadi G, Or R. Graft vs autoimmunity following allogeneic non-myeloablative blood stem cell transplantation in a patient with chronic myelogenous leukemia and severe systemic psoriasis and psoriatic polyarthritis. Exp Hematol 2000; 28: 853-7.

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Cord blood-derived hematopoietic progenitor cells: in vitro response to hematopoietic growth factors and their recruitment into the S-phase of the cell cycle

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ABSTRACT

Background and Objectives. **In the recent years many studies on the expansion of cord blood (CB)-derived progenitor cells have been performed, whereas less information is available on their cycling status. The objective of this study was to evaluate the cycling status of CB-derived colony-forming cells (CFC) and long-term culture-initiating cells (LTC-IC), and their recruitment into the S-phase of the cell cycle in response to a combination of cytokines.**

Design and Methods. **CB-derived CFC and LTC-IC were first quantified by standard clonogenic assay and long-term culture, respectively. In a second set of experiments, CB-derived progenitor cells were incubated with interleukin(IL)-3, stem cell factor (SCF) and granulocyte colony-stimulating factor (G-CSF) and their cell cycle status assessed both by the cytosine arabinoside (Ara-C) suicide approach and by flow cytometric DNA analysis.**

Results. **We found that only small proportions of both CFC and LTC-IC were in the S-phase of the cell cycle. These estimates were confirmed by flow cytometric DNA analysis, which showed that 96%±2% of CB-derived CD34+ cells were in G_0/G_1 and only 1.6%±0.4% in the S-phase. Staining of CD34+ cells with an anti-statin monoclonal antibody, a marker of the G_0 phase, indicated that among CD34+ cells with a flow cytometric DNA content typical of the G_0/G_1 phase, 68%±7% of cells were in the G_0 phase of the cell cycle. Twenty-four hour incubation with IL-3, SCF and G-CSF significantly increased the proportion of cells in the S-phase for both CFC and LTC-IC without inducing any loss in their number. Flow cytometric DNA analysis also showed an increase of CD34+ cells in the S-phase upon continuous exposure to these cytokines.**

Interpretations and Conclusions. **Our findings indicate that: i) a small number of CB-derived CFC and LTC-IC are in the S-phase of the cell cycle; ii) a substantial number of CD34+ cells with a flow cytometric DNA content typical of the G_0/G_1 fraction are cycling, as they are found in the G_1 phase of the cell cycle; iii) 24-hour incubation with IL-3, SCF and G-CSF can drive a proportion of progenitor cells into the S-phase without reducing their number.**

Key words: $CD34^+$, cell cycle, CFC/LTC-IC, statin

In the recent years umbilical cord blood (CB) has been regarded as an attractive source of hematopoietic progenitor cells for bone marrow transplantation.[1] However, its use for this purpose has been limited mainly to pediatric patients[2, 3] because of the low number of progenitor cells which can be detected in each single unit of CB.[4] As a consequence, over the last few years, many studies have been carried out with the aim of establishing a combination of cytokines which would allow *ex vivo* expansion of the more primitive hematopoietic progenitor cells[5,6] in order to make this source of hematopoietic progenitor cells available for adult patients as well. However, no definitive agreement on a common expansion protocol has so far been reached. In contrast to the large body of studies on the expansion of CB progenitor cells, less information is currently available on the cycling status of CB-derived hematopoietic progenitor cells, both committed (colony-forming cells, CFC) and early (long-term culture-initiating cells, LTC-IC). The definition of the cycling status of these progenitor cells can be of interest for different reasons. For instance, retroviral gene transfer requires the target cells to be cycling: in this regard, CB-derived progenitor cells could be an appealing source of cells for gene transfer protocols,[7] provided they are in the S-phase of the cell cycle. Should these cells be in a quiescent status, they would need to be driven into the cell cycle in order to achieve good efficiency in gene transfer. Moreover, in recent years the cycling status of both CFC and LTC-IC derived from different sources (bone marrow, peripheral blood and

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apheretic products obtained after mobilization protocols with growth factors and/or chemotherapy) has been defined[8-11] whereas it has not yet been definitely clarified for CB-derived hematopoietic progenitor cells.

We have evaluated the cycling status of both CFC and LTC-IC derived from umbilical CB using the Ara-C suicide technique which is based on the capacity of cytosine arabinoside to selectively kill cells which are in the S-phase of the cell cycle.[12] We found that a small number of CB-derived hematopoietic progenitor cells is in the S-phase of the cell cycle. We have also confirmed these results by flow cytometric analysis of the DNA content of $CD34^+$ cells and determined the proportion of these cells in the different phases of the cell cycle (including the G_0 by means of an anti-statin monoclonal antibody). Finally, we have found that the combination of interleukin-3 (IL-3), stem cell factor and granulocyte colony-stimulating factor can trigger most CFC and LTC-IC into the S-phase of the cell cycle within 24 hours of incubation.

Design and Methods

Cells and cell separation procedures

Cord blood was obtained from normal full-term deliveries, after informed consent. Mononuclear light-density cord blood cells (LDCBCs) were obtained by centrifugation on a Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) gradient (1,077 g/mL) for 30 minutes at 400 g. After washing, interface cells were resuspended in Iscove's modified Dulbecco's medium (IMDM, Irvine Scientific, Santa Ana, CA, USA) and counted using a Neubauer hemocytometer. The selection of CD34+ cells was carried out using the MiniMacs magnetic cell sorting-device (Miltenyi Biotec GmbH, Germany), as previously described.[13] The purity of the selected CD34+ cells was on average > 95% and the recovery on average ~ 60%.

Growth factors

Highly purified recombinant human interleukin-3 (IL-3) and recombinant human granulocyte-colony-stimulating factor (G-CSF) were kindly provided by Sandoz International (Basel, Switzerland); recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) and recombinant human stem cell factor (SCF) by PeproTech Inc. (Rocky Hill, NJ, USA) Recombinant human erythropoietin (Epo) was obtained from Boehringer Mannheim (Mannheim, Germany).

Cytosine arabinoside (Ara-C) suicide assay

LDCBCs were incubated in IMDM with 20% fetal bovine serum (FBS; HyClone, Logan, UT) or in IMDM containing $5x10^{-5}$ mol/L β-mercaptoethanol (Sigma Chemicals, Milan, Italy), 10 mg/mL human insulin (Sigma), 200 mg/mL iron-saturated human transferrin (ICN Pharmaceuticals, Costa Mesa, CA, USA), 20 mg/mL of deionized bovine serum albumin (StemCell Technologies, Vancouver, BC, Canada), with or without 100 ng/mL SCF (PeproTec Inc.), 20 ng/mL IL-3 (Sandoz) and 20 ng/mL G-CSF (Sandoz). Equal volumes of cell suspension were incubated at 37°C, 5% CO_2 in air for 24 hours, in the presence or absence of 10^{-6} mol/L Ara-C. This concentration of Ara-C was chosen on the basis of preliminary experiments carried out in our laboratory and according to published data,[11,14] showing that a plateau of specific killing (with a minimal aspecific toxic effect) was reached at concentrations ranging between $1\text{-}2x10^{-6}$ mol/L. The cells were then transferred into a tube, washed twice with fresh medium and resuspended in IMDM, and appropriate quantities from each sample were assayed for LTC-IC and CFC. The proportions of CFC and LTC-IC killed by Ara-C (i.e., the proportions of progenitor cells in the S-phase) were then calculated.

Clonogenic assay

Clonogenic assays were performed as described elsewhere,[15] with minor modifications. Briefly, $2x10^4$ LDCBCs were plated in 35-mm Petri dishes in 1 mL aliquots of IMDM containing 30% FBS (HyClone), $5x10^{-5}$ mol/L β-mercaptoethanol, 0.9% (w/v) methylcellulose, GM-CSF, IL-3 (10 ng/mL each factor), SCF (50 ng/mL) and 3 IU/mL erythropoietin. After 14 days of incubation at 37°C and 5% CO_2, the number of colonies was scored using an inverted microscope.

LTC-IC assay

For this assay, 3×10^6 LDCBCs were resuspended in 2.5 mL of myeloid long-term culture (LTC) medium (StemCell Technologies) supplemented just prior to use with 10^{-6} mol/L freshly dissolved hydrocortisone sodium hemisuccinate (Sigma) and plated in 35 mm Petri culture dishes onto a pre-established feeder layer of $3x10^5$ irradiated (8,000 cGy) M210B4 fibroblasts[16] (kindly provided by Dr. CJ Eaves). The cultures were maintained at 37°C and 5% CO_2 for 5 weeks with weekly replacement of half the medium and non-adherent cells with fresh LTC medium. At the end of the 5 weeks, all of the non-adherent cells were removed and combined with the cells harvested from the adherent fraction by trypsinization.[17] These cells were then washed and aliquots were assayed for their CFC content in a clonogenic assay as described above. The number of LTC-IC present in the initial sample (per 10^6 mononuclear cells) was calculated as reported.[13,18]

Incubation of CD34+ cells with growth factors

For the assessment of the progression through the cell cycle, $1x10^5$/mL CD34+ cells (purity > 95%) were incubated in Iscove medium containing $5x10^{-5}$ mol/L β-mercaptoethanol, 10 mg/mL human insulin, 200 mg/mL iron-saturated human transferrin, 20 mg/mL of deionized bovine serum albumin in the presence of 100 ng/mL SCF, 20 ng/mL IL-3 and 20 ng/mL G-CSF. After 6, 12 and 24 hours aliquots of cells were analyzed for their DNA content as described below.

Immunocytochemical detection of statin

Statin was detected on cytocentrifuge preparations of freshly harvested CD34+ cells by an immuno alkaline-phosphatase method (Streptavidin-biotin complex, LSAB2 kit, Dakopatts). Briefly, cells were fixed in 70% ethanol at -20°C for 20 min and rehydrated in PBS. After permeabilization of the cells with PBS/Tween/BSA solution for 10 min, slides were incubated in a moist chamber at room temperature with the anti-statin monoclonal antibody S-44 (kindly provided by Dr. E. Wang), diluted 1:200 for 12 hours. After washing with PBS, they were incubated with a biotinylated anti-mouse rabbit Ig and then with the phosphatase alkaline/streptavidine complex. After washing, slides were stained with the following medium: naphthol-AS-BI phosphate (50 mg), dimethylformamide (0.6 mL), Tris HCl pH 8.2 0.05 mmol/L (100 mL), levamisole 1 mol/L, sodium nitrite 4% (0.5 mL) and New Fuchsin 5% (0.2 mL) for 15 min. Slides were finally washed and counterstained with Mayer's Hemalum for 5 min.

Immunofluorescent detection of statin, propidium iodide DNA staining and flow cytometry

For flow cytometric analysis, CD34+ cells were first fixed in 70% cold ethanol at 4°C for at least 30 minutes and then rehydrated in PBS, treated for 20 minutes with 5% normal goat serum in PBS and permeabilized with PBS/Tween/BSA solution for 10 minutes at room temperature in a moist chamber with 1:200 dilution in PBS of the anti-statin monoclonal antibody, S-44.[19,20] Cells were then washed for 10 min in PBS and incubated with a 1:50 dilution of a phycoerythrin (PE)-conjugated goat anti-mouse IgG (Sigma Chemical) in PBS/Tween/BSA solution. For DNA staining, a previously described single step procedure on a separate sample of ethanol-fixed cells was employed.[21] Flow cytometric determinations, for both statin-positivity and DNA content, were made with a Becton Dickinson FACStar flow cytometer, under the conditions previously described.[22]

Statistical methods

Results are expressed as mean ± standard deviation (SD). Student's t-test for paired data was used to test the probability of significant differences between samples; all data were analyzed using the statistical package Statview 4.02 (BrainPower Inc., Calabasas, CA, USA) run on a iMac personal computer (Apple Computer Inc, Cupertino, CA, USA).

Results

Cell cycle status of CFC

After incubation for 24 hours in liquid culture in the presence of FBS with or without 10^{-6} M Ara-C CB-derived mononuclear cells (n=7) were plated in methylcellulose and the number of CFC assessed. As shown in Table 1, the proportion of CFC killed by Ara-C (corresponding to the proportion of CFC in the S-phase of the cell cycle) was < 20%, suggesting that the great majority of CB-derived CFCs did not enter the S-phase within the 24- hour incubation. This was also true when the single subtypes of hematopoietic progenitors (CFU-GM and BFU-E) were considered. In order to rule out the possibility that the entry of hematopoietic progenitor cells into the S-phase could be inhibited by the presence of significant amount of TGFβ1 contained in FBS,[23, 24] we also performed a 24-hour Ara-C incubation in serum-free culture. The killing of CFC by Ara-C was not statistically different between serum-free cultures and FBS-containing cultures (data not shown), indicating that no effect on the cell cycle was exerted by TGFβ1 or by other similar inhibitory components present in the FBS.

Table 1. Number of CFC and LTC-IC after 24-hour incubation with FBS and after exposure to IL-3, SCF and G-CSF (GF) in serum-free (SF) culture. The proportion of progenitor cells in the S-phase of the cell cycle is also shown.

Culture conditions	*BFU-E**	*CFU-GM**	*CFC**	*LTC-IC**
24 hours FBS	22±5	12±4	36±8	23±16
24 hours FBS + Ara-C	18±3^	10±2^	30±7^	19±7^
% of cells in the S-phase	24±7	2±2	18±5	17±9
24 hours SF + GF	24±13	10±7	37±11	22±9
24 hours SF + GF + Ara-C	8±5#	3±1#	11±8#	4±3#
% of cells in the S-phase after GF	66±4	68±7	71±4	81±7

** Progenitor cell numbers are expressed per $2x10^4$ CB mononuclear cells or per 1x106 CB mononuclear cells for CFC (n=7) and LTC-IC (n=4), respectively. Values shown are means ±SD. ^ Compared to 24 hours FBS, p > 0.05 (Student's t-Test for paired values). # Compared to 24 hours SF+GF, p < 0.05 (Student's t-Test for paired values).*

Kinetics of activation of CFC into the S-phase

The kinetics of recruitment of CB-derived CFC into the S-phase of the cell cycle was studied using two different types of experiments. First, we incubated CB-derived mononuclear cells under the same conditions (IMDM +20% FCS with or without Ara-C) used for the 24-hour Ara-C suicide but for a longer period of time (up to 36 hours) and then we assessed the number of surviving CFC after plating in methylcellulose. We found that 41±11% of CFC were killed by Ara-C after 36 hours of incubation, indicating that a substantial proportion of CFC entered the S-phase after the first 24 hours, during which no significant killing was observed (Figure 1). In a second set of experiments mononuclear cells were incubated for up to 36 hours in a serum-free medium containing SCF, IL-3 and G-CSF with or without Ara-C. This approach was chosen because in previous experiments[8] this combination of cytokines has been shown to trigger most of the quiescent population of CFC and LTC-IC derived from the peripheral blood into the S-phase without any significant loss of their number. Under these conditions we observed that after 24 hours 69% of CFC had entered the S phase and that within 36 hours more than 90% of the CFC were recruited into the S-phase (Table 1 and Figure 1). We also found that, at least after 24 hours of incubation with this combination of cytokines (and in the absence of Ara-C), there was no significant loss of the number of CFC compared to their input number, suggesting that, as for their peripheral blood counterpart, also for CB-derived CFC IL-3, SCF and G-CSF can trigger cell proliferation without inducing a substantial amount of differentiation (Table 2).

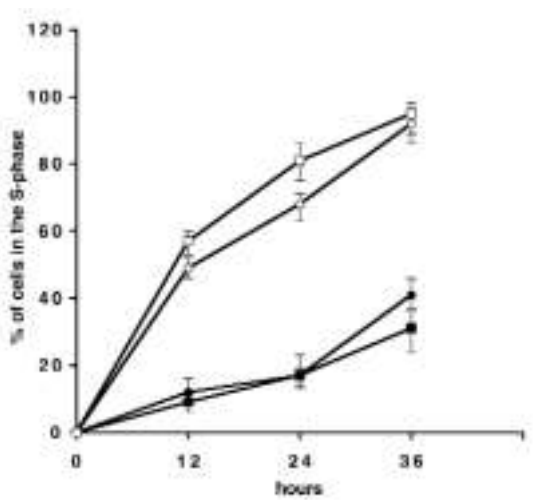

Figure 1. Kinetics of recruitment of CB-derived CFC and LTC-IC into the S-phase. Solid symbols indicate the percentage of CFC (circles) and LTC-IC (squares) in the S-phase of the cell cycle upon continuous liquid culture in the presence of 20% FCS. Open symbols indicate the percentage of CFC (circles) and LTC-IC (squares) upon continuous liquid culture in a serum free medium in the presence of IL-3, SCF and G-CSF. Each point represents the mean±SD of three different experiments.

Cell cycle status of LTC-IC and kinetics of activation into the S-phase

The cycling status of CB-derived LTC-IC (n=4) was also examined, using the same strategy as that for the CFC. In 4 different experiments in which mononuclear cells were incubated for 24 hours in liquid cultures containing IMDM and 20% FCS with or without Ara-C, we observed 17%±9% more killing than in control cultures (Table 1); a similar result was obtained when incubation with Ara-C was performed in serum-free conditions. Thus, these data clearly indicate that CB-derived LTC-IC are not in the S-phase, similarly to their committed counterpart. The same combination of cytokines and the same time course experiments used for CFC were also used for studying the kinetics of progression of the LTC-IC into the S-phase. Table 1 shows that after 24 hours of incubation with IL-3, SCF and G-CSF more than 80% of LTC-IC were killed by Ara-C and that within 36 hours > 95% of LTC-IC had entered the S-phase of the cell cycle (Figure 1). As for CFC, we found that this combination was able to sustain, at least for 24 hours, the number of LTC-IC at the input level (Table 2) while recruiting most of the LTC-IC into the S-phase of the cell cycle.

Flow cytometric analysis of cell cycle phase distribution and statin expression of CB-derived CD34⁺ cultured cells

With DNA flow cytometry, most freshly harvested CB-derived $CD34^+$ cells were in the G_0/G_1 phase of the cell cycle, whereas only 1.6±0.4% were in the S-phase and 2.4±2.3% in the G_2/M phase thus confirming that most of CB-derived

Table 2. Changes in the number of CFC and LTC-IC during 24 hours of incubation with different types of media. Progenitor cell numbers are expressed as a percentage (±SD) of the number detected at the beginning of the incubation (input cells). Increases or decreases of the cell numbers are not statistically significant (Student *t*-Test: $p > 0.05$).

	BFU-E	*CFU-GM*	*CFC*	*LTC-IC*
*% of input cells after 24 h incubation with 20% FCS (without Ara-C)**	85±6	81±9	83±10	91±12
*% of input cells after 24 h incubation in SF with GF (without Ara-C)**	108±30	77±15	88±9	92±18

**Results are expressed as a mean ± SD of 7 (for CFC) and 4 (for LTC-IC) different experiments.*

Table 3. Cell cycle distribution of freshly harvested CD34+ cells derived from cord blood.

	G0	*G1*	*S*	*G2/M*
% of CD34+ cells	68.4±7	27.6±2	1.6±0.5	2.4±2

Results are expressed as a mean ± SD of 7 different experiments. G0 cells are calculated on the basis of the percentage of CD34+ cells expressing the nuclear protein statin.

Table 4. Cell cycle distribution of CB-derived cultured CD34+ cells after incubation with IL-3, G-CSF and SCF.*

	% G_0/G_1	*% S*	*% G_2/M*
freshly harvested	95±6	1.8±0.8	3.2±1
6 hours	77.4±8	11±3	11.6±2
12 hours	82.4±6	9.1±2	8.5±3
24 hours	81±5	8.5±3	11.5±4

**CD34+ cells were incubated ia a serum-free medium in the presence of IL-3, G-CSF (20 ng/mL each factor) and SCF (100 ng/mL) and the DNA content assessed by cytofluorimetric assay after 6, 12 and 24 hours of incubation. Results are expressed as a mean ±SD of 3 experiments.*

progenitor cells are in the G_0/G_1 phase (Table 3). To investigate the cell cycle distribution of freshly harvested CD34+ cells further, these cells were stained with the PE-conjugated anti-statin monoclonal antibody S-44 and the proportion of fluorescent cells evaluated by means of a FACStar flow cytometer. Expression of statin, a 57-kDa nuclear protein, has been recognized as an unique marker of quiescent cells:[19] the protein is found in resting (G_0) cells and is rapidly down-regulated when cells progress to the G_1 phase. Thus, expression of statin can be used to identify cells which are in the G_0 phase of the cell cycle: this can be assessed both by immunocytochemistry and by flow cytometry. Using the latter, we found that 68.4±7% of freshly harvested CD34+ cells expressed statin (n=7), indicating that about 2/3 of CD34+ cells in the G_0/G_1 region of the flow cytometric DNA histograms are in the G_0 phase and the remaining are in the G_1 phase of the cell cycle (Table 3). We also assessed the proportion of CD34+ cells in the different phases of the cell cycle at different time points (after 6, 12 and 24 hours) during incubation in a serum-free medium in the presence of IL-3, SCF and G-CSF. As shown in Table 4 a discrete proportion of CD34+ cultured cells had rapidly progressed into the S-phase already after 6 hours of incubation (11%) and a similar proportion was also detectable after 12 and 24 hours of incubation (9.1% and 7.5% respectively). Importantly, the proportion of CD34+ cells did not change significantly during the 24-hour culture (97.3±2% and 95.5±3% at the beginning and after 24 hours of incubation with growth factors, respectively; $p>0.05$) confirming that the changes observed in the cell cycle distribution were effectively dependent on the progression through the cell cycle of CD34+ cells. We also observed an increase in the absolute number of CD34+ cells after the 24-hour incubation but it did not reach a statistical significance. This last observation suggests that although recruited into the S-phase within 24 hours of culture, CD34+ cells require a longer time to complete cell division.

Discussion

The objective of this study was to evaluate the cycling status of CB-derived progenitor cells and their kinetics of recruitment into the S-phase of the cell cycle in response to a combination of different hematopoietic growth factors. We studied both the committed progenitor cells (CFU-GEMM, CFU-GM and BFU-E, all together defined as CFC) and the early progenitor LTC-IC which up to now are considered the most immature progenitor cells which can be investigated *in vitro*.[25] In fact, whereas the CFC are responsible for the short-term engraftment of a transplant, the LTC-IC are thought to represent a more reliable approximation of the hematopoietic stem cells responsible for long-term engraftment. The proportion of cells in the S-phase of the cell cycle was determined using the Ara-C suicide technique which has been shown to give reliable and reproducible results when compared to the ^{3}H thymidine incorporation suicide test.[12,14] Our results show that after 24 hours of liquid culture in the presence of FCS there was no statistical difference between the number of CFC and LTC-IC grown in the presence or absence of Ara-C, clearly indicating that very few committed and early CB-derived progenitor cells are in the S-phase of the cell cycle. The same results were observed when similar experiments were performed in serum-free conditions, ruling out the possibility that the presence of FBS could have affected the cycling status of the progenitor cells.[23, 24] Our results on the proportions of CFC and LTC-IC in the S-phase confirm those of a recent study in which the ^{3}H thymidine incorporation suicide technique was used instead of the Ara-C suicide technique[26] demonstrating that the Ara-C approach is comparable to thymidine suicide for the assessment of the proportion of hematopoietic progenitor cells in the S-phase of the cell cycle. These data were further strengthened by our finding that 96% of the CD34+ cells derived from CB were found in the G_0/G_1 phase of the cell cycle and only 1.6% in the S-phase,

according to the flow cytometric assessment of the DNA content. By means of this technique, however, it is not possible to distinguish, among the cell fraction with a DNA content typical of the G_0/G_1 phase, cells which are really quiescent (G_0) from cells which are cycling (G_1). Thus, we took advantage of an anti-statin monoclonal antibody in order to discriminate between resting (statin positive) and cycling (statin negative) cells. We found, in fact, that about 60% of freshly harvested CB-derived $CD34^+$ cells were resting, being out of the cell cycle, whereas most of the remaining progenitor cells was actually cycling, being in the G_1 phase of the cell cycle. The fact that incubation of progenitor cells in the presence of FCS for 36 hours was associated with the progression into the S-phase of a significant number of both CFC and LTC-IC can be interpreted considering that these cells entering the S-phase derive from those in the G_1 phase of the cell cycle. Our data are substantially in agreement with previous reports which showed, by means of the evaluation of DNA content by flow cytometry, that more than 90% of $CD34^+$ cells derived from CB are in the G_0/G_1 phase of the cell cycle.[27-29] However, due to the technical approach which was used in these studies, the whole $CD34^+$ cell population rather than the single types of progenitor cells were evaluated. Most importantly, we have been able, for the first time, to discriminate between the proportion of these cells in the G_0 and G_1 phase before treatment with cytokines.

A new observation of our work emerges from the evaluation of the kinetics of recruitment of CB-derived progenitor cells into the S-phase of the cell cycle. To study the kinetics of recruitment into the S-phase, we incubated the mononuclear cell fraction derived from CB with a combination of cytokines and then assessed the proportion of CFC and LTC-IC entering the S-phase of the cell cycle after 12, 24 and 36 hours exposure to these cytokines. In these experimental conditions we observed that 70% and 81% of CFC and LTC-IC respectively entered the S-phase of the cell cycle after 24 hours. Furthermore, more than 90% of both progenitor cells were killed by Ara-C upon continuous exposure to G-CSF, IL-3 and SCF (up to 36 hours) suggesting that a proportion of both CFC and LTC-IC can be rapidly recruited into the S-phase of the cell cycle *in vitro* in the presence of these cytokines. Cytofluorimetric assessment of the DNA content confirmed that CB-derived $CD34^+$ cells in the presence of this combination of cytokines rapidly start to progress into the S-phase. Interestingly, our data showing that incubation of CB progenitor cells in serum-free culture and hematopoietic growth factors is associated with a more rapid induction of the cell cycle in comparison to incubation in the presence of FBS is in keeping with results reported by Jordan and coworkers[30] who found that serum-free conditions are preferable for activating quiescent cells.

Taken together our results are in apparent contrast with parallel experiments performed with bone marrow (BM)- and peripheral blood (PB)-derived progenitor cells which showed that a longer exposure to these cytokines (up to 48-72 hours) is needed to trigger such cells into the cell cycle.[8] The difference between the kinetics of recruitment into the cell cycle of CB and adult progenitor cells can be explained assuming that the rate of exit from the G_0/G_1 phase in response to cytokines is faster for at least a proportion of CB-derived progenitor cells than for PB- or BM-derived progenitor cells.[27] We do not have a definitive explanation for this different behavior but we can formulate two not mutually exclusive interpretations: i) CB-derived progenitor cells may respond differently to hematopoietic growth factors: in fact, it has been shown that the proliferative response of CB-derived CFC and LTC-IC to SCF is higher than that of their PB- or BM- derived counterpart;[29,31,32] ii) at least a proportion of the progenitor cells rapidly recruited into the S-phase is derived from those in the G1 phase of the cell cycle, which represent a substantial number of CB-derived $CD34^+$ cells, as shown by our experiments performed with statin.

We also found that the absolute number of CFC and LTC-IC after 24 and 36 hours of exposure to IL-3, SCF and G-CSF (in the absence of Ara-C) was not statistically different from their baseline (at the beginning of incubation) number. Moreover, we found that differentiation of cultured $CD34^+$ cells, if any, is minimal. In fact, neither their proportion nor absolute number changed significantly after 24 hours of incubation in the presence of growth factors. This suggests that the combination of cytokines used in our experiments is not only able to trigger the cell cycle of CB-derived progenitor cells but also does not induce a decline in their number (possibly due to their differentiation or death), at least for 24 hours. This latter observation could have a practical implication for example when CB-derived hematopoietic progenitor cells should be used as a target for retroviral mediated gene transfer protocols.

Finally, our results can also be interpreted considering what has been previously reported by Gothot *et al.*[33] who found that in steady-state bone marrow the turnover of primitive hematopoietic cells is lower than that of their committed counterpart and that a direct relationship exists between the rate of cycling and

the degree of lineage commitment. In freshly harvested CB we found that very few LTC-IC are in the S-phase of the cell cycle; however, in contrast to that which was observed in the bone marrow, also very few CFC were found in the S-phase of the cell cycle. A possible explanation is that a different regulation exists for bone marrow hematopoiesis and for CB hematopoiesis, but the factors which could determine this difference are presently unknown. Also, these authors found that cultured $CD34^+$ cells re-entering G_0 have a shorter pre-replicating phase than freshly isolated G0 $CD34^+$ cells. This observation could explain the high proportion (up to 90%, Figure 1) of LTC-IC and CFC entering the S-phase when incubated in the presence of cytokines for 36-48 hours.

In summary, our data show that very few CB-derived CFC and LTC-IC are in the S-phase of the cell cycle. Furthermore, results on cell cycle distribution and statin expression of $CD34^+$ cells, while confirming the low numbers of progenitor cells in the S-phase of the cell cycle, clearly indicate that a significant number of $CD34^+$ cells are in the G_1 phase of the cell cycle. Our results also provide new information on the kinetics of recruitment into the S-phase of the cell cycle of CB-derived CFC and LTC-IC, and on culture conditions which can modify their cycling status without reducing their numbers. These findings could play an important role in the engineering of CB-derived progenitor cells for gene therapy and in the designing of clinically relevant protocols for their *ex vivo* expansion.

References

1. Gluckman E. The therapeutic potential of fetal and neonatal hematopoietic stem cells. N Engl J Med 1996; 335:1839-40.
2. Gluckman E, Broxmeyer HE, Auerbach AD, et al. Hematopoietic reconstitution in a patient with Fanconi`s anemia by means of umbilical-cord blood from an HLA-identical sibling. N Engl J Med 1989; 321: 1174-8.
3. Kurtzberg J, Laughlin M, Graham ML, et al. Placental blood as a source of hematopoietic stem cells for transplantation into unrelated recipients. N Engl J Med 1996; 335:157-70.
4. Broxmeyer HE, Hangoc C, Cooper S, et al. Growth characteristics and expansion of human umbilical cord blood and estimation of its potential for transplantation in adults. Proc Natl Acad Sci USA 1992; 89:4109-13.
5. Piacibello W, Sanavio F, Garetto L, et al. Extensive amplification and self-renewal of human primitive hematopoietic stem cells from cord blood. Blood 1997; 89:2644-53.
6. Zandstra PW, Conneally E, Piret PM, Eaves CJ. Ontogeny-associated changes in the cytokine responses of primitive human hematopoietic cells. Br J Haematol 1998; 101:770-8.
7. Kohn DB, Weinberg KI, Nolta JA, et al. Engraftment of gene-modified umbilical cord blood cells in neonates with adenosine deaminase deficiency. Nature Med 1995; 1:1017-23.
8. Ponchio L, Conneally E, Eaves C. Quantitation of the quiescent fraction of long term culture initiating cells in normal humanblood and marrow and the kinetics of their growth factor-stimulated entry into S-phase in vitro. Blood 1995; 86:3314-21.
9. Ponchio L, Eaves C, Hogge D, et al. Both cycling and noncycling primitive progenitors continue to be mobilized into the circulation during the leukapheresis of patients pretreated with chemotherapy and G-CSF. Br J Haematol1995; 99:394-402.
10. Danova M, Aglietta M. Cytokine receptors, growth factors and cell cycle in human bone marrow and peripheral blood hematopoietic progenitors. Haematologica 1997; 82:622-39.
11. Lemoli R, Tafuri A, Fortuna A, et al. Cycling status of CD34+ cells mobilized into peripheral blood of healthy donors by recombinant human granulocyte colony stimulating factor. Blood 1997; 89:1189-96.
12. Dresch C, El Kebir N, Metral J, Karsdorf, A. Cytosine arabinoside as a suicide agent for human colony forming cells. Exp Hematol 1983; 11:187-92.
13. Lucotti C, Malabarba L, Rosti V, et al. Cell cycle distribution of cord blood-derived heamatopoietic progenitor cells and their recruitment into the S-phase of the cell cycle. Br J of Haematol 2000; 108:621-8.
14. Preisler HD, Epstein J. A comparison of two methods for determining the sensitivity of human myeloid colony-forming units to cytosine arabinoside. Br J Haematol 1981; 47:519-27.
15. Carlo Stella C, Cazzola M, Ganser A, et al. Synergistic antiproliferative effect of recombinant interferon γ with recombinant interferon a on chronic myelogenous leukemia haematopoietic progenitor cells (CFU-GEMM, CFU-Mk, BFU-E, CFU-GM). Blood 1988; 72: 1293-9.
16. Lemoine FM, Humphries MK, Abraham SDM, Kristal G, Eaves CJ. Partial characterization of a novel stromal cell-derived pre-B-cell growth factor active on normal and immortalized pre-B cells. Exp Hematol 1988; 16: 718-25.
17. Sutherland HJ, Eaves CJ, Eaves AC, Dragowska W, Lansdorp PM. Characterization and partial purification of human marrow cells capable of initiating long term haematopoiesis in vitro. Blood 1989; 74:1563-9.
18. Sutherland HJ, Eaves CJ. Long-term culture of human myeloid cells. In: Culture of Hematopoietic cells (ed. by Freshney RI, Pragnell IB and Freshney MG), New York, 1994, Wiley, 139-62.
19. Wang E. A 57.000-mol-wt protein uniquely present in non-proliferating cells and senescent human fibroblasts. J Cell Biol 1985; 100:545-51.
20. Pellicciari C, Mangiarotti R, Bottone MG, Danova M, Wang E. Identification of resting cells by dual parameter flow cytometry of statin expression and DNA content. Cytometry 1995; 21:329-37.
21. Rosti V, Bergamaschi G, Lucotti L, et al. Oligodeoxynucleotides antisense to c-abl specifically inhibit entry into S-phase of CD34+ hematopoietic cells end their differentiation to granulocyte-macrophage progenitors. Blood 1995; 86:3387-93.
22. Mangiarotti R, Bottone MG, Danova M, Pellicciari C. Bivariate flow cytometric analysis of DNA content versus immunopositivity for ribonucleotide reductase M1 subunit in the cell cycle. Cytometry 1998; 32:78-85.
23. Cashman JD, Eaves AC, Raines EW, et al. Mechanisms that regulate the cell cycle status of very primitive hematopoietic cells in long term human marrow cultures. I. Stimulatory role of a variety of mesenchi-

mal cell activators and inhibitory role of TGFβ1. Blood 1990; 75:96-101.
24. Sitnicka E, Ruscetti FW, Priestle GV, Wolf NS, Bartelmez SH. Transforming growth factor b1 directly and reversibly inhibits the initial cell divisions of long term repopulating hematopoietic stem cells. Blood 1995; 88:82-8.
25. Sutherland HJ, Lansdorp PM, Henkelman JC, Eaves AC, Eaves JC. Functional characterization of individual human hematopoietic stem cells cultured in limiting dilution on supportive marrow stromal layers. Proc Natl Acad Sci USA 1990; 87:3584-8.
26. Movassagh M, Caillot L, Baillou C, Guigon M, Lemoine FM. Optimization of the cycling of clonogenic and primitive cord blood progenitor by various growth factors. Stem Cells 1997; 15:214-22.
27. Traycoff CM, Abboud MR, Laver J, Wade Clapp D, Srour EF. Rapid exit from G_0/G_1 phases of cell cycle in response to stem cell factor confers on umbilical cord blood CD34+ cells an enhanced ex vivo expansion potential. Exp Hematol 1994; 22:1264-72.
28. Hao QL, Shah AJ, ThiemannFT, Smogorzewska EM, Crooks GM. A functional comparison of CD34+ CD38– cells in cord blood and bone marrow. Blood 1995; 86:3745-53.
29. Roberts AW, Metcalf D. Noncycling status of peripheral blood progenitor cells mobilized by granulocyte colony-stimulating factor and other cytokines. Blood 1995; 86:1600-5.
30. Jordan CT, Yamasaki G, Minamoto D. High-resolution cell cycle analysis of defined phenotypic subsets within primitive human hemopoietic cell populations. Exp Hematol 1996; 24:1347-55.
31. Tanaka R, Katayama N, Ohishi K, et al. Accelerated cell-cycling of hematopoietic progenitor cells by growth factor. Blood 1995; 86:73-9.
32. Weeks SFA, Van Bockstaele DR, Plum J, et al. CD34++CD38– and CD34+CD38+ human hematopoietic progenitors from fetal liver, cord blood, and adult bone marrow respond differently to hematopoietic cytokines depending on the ontogenic source. Exp Hematol 1998; 26:1034-90.
33. Gothot A, Pyatt R, McMahel J, Rice S, Srour EF. Functional heterogeneity of human CD34+ cells isolated in subcompartments of the G0/G1 phase of the cell cycle. Blood 1997; 90:4384-92.

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Cord blood transplantation in childhood

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The first allogeneic umbilical cord blood transplant (UCBT) was successfully performed in 1988 to treat a boy with Fanconi's anemia; the umbilical cord blood donor was his HLA identical sister.[1] Twelve years later, this patient is doing well with full donor hematopoietic and lymphoid reconstitution.

Since then, our knowledge of the biological characteristics of umbilical cord blood cells has increased, emphasizing the advantages of using umbilical cord blood stem cells for transplantation. Simultaneously, umbilical cord blood banks have been established world-wide for both related and unrelated umbilical cord blood transplants,[2-4] with more than 40,000 units currently available and more than 1,500 UCBT having been performed mainly in children and less frequently in adults, for either malignant or non malignant disorders. Several reports on these transplants have been published in the last 5 years.[5-10]

The main practical advantages of using umbilical cord blood as an alternative source of stem cells are the relative ease of procurement, the absence of risks to donor, the reduced risk of transmitting viral infections (Cytomegalovirus, Epstein-Barr virus, etc.) and, especially for transplant from unrelated donors, the prompt availability of cryopreserved samples. The use of stored cord blood units is certainly capable of reducing the time required to perform an allogeneic transplant of hematopoietic stem cells, which at present can be estimated at an average of 4-5 months for bone marrow transplantation (BMT) from unrelated individuals. On average, the time that elapses between opening the search and identifying a unit of placental blood usable to perform the transplant is no more than 20 days. This reduction is of particular interest for patients affected by acute leukemia or by life-threatening diseases (severe combined immune deficiencies, malignant osteopetrosis, hemophagocytic lymphohistiocytosis, etc.), which lead to an unstable hematologic or clinical balance.

It has been recently and formally proved that UCBT recipients are exposed to a lower incidence and a lower severity of graft-versus-host disease (GVHD).[11,12] In particular, one study compared children given UCBT with others receiving bone marrow transplantation from a compatible relative and unequivocally showed that use of the former source of hematopoietic cells led to a statistically significant reduction in the incidence and severity of both acute and chronic GVHD.[11] The reduced incidence of chronic GVHD is of particular interest considering the detrimental impact played by this complication on a growing organism.

The biological reasons for the reduction of GVHD after UCBT are still not precisely defined. One of the first hypothesis formulated to explain the reduced incidence and severity of GVHD in UCBT recipients was that these patients receive a significantly lower number of T-cells infused per kg of recipient body weight as compared to subjects given BMT (approximately 1 log less than a standard allogeneic marrow transplant). However, the study published by the *New York Cord Blood Bank*[8] documented that younger children given UCBT were exposed to a lower risk of GVHD than older patients. This finding strongly argues against the speculation that the reduced rate of GVHD is mainly due to the low number of T-cell infused, since younger patients received the highest T-cell doses.

The combination of several immunologic properties and peculiarities of cord blood lymphocytes are likely responsible for the reduction of GVHD observed in UCBT recipients.[13] In fact, cord blood lymphocytes are naive (as proved by the higher number of cells expressing the RA isoform of the CD45 molecule), produce lower amounts of pro-inflammatory cytokines such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α, are characterized by a lower frequency of interleukin (IL)-2, IFN-γ and TNF-α producing cells and display absent or markedly

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reduced responsiveness to allogeneic stimuli in a secondary mixed lymphocyte reaction (MLR).[14-17] Alloantigen-specific T-cells with suppressor phenotype have been suggested to exist among cord blood lymphocytes and they might play a role in the reduction of the risk of GVHD associated with UCBT.[18]

Recently, attention has also been paid to the study of cord blood antigen-presenting cells (APC). These fundamental cells of the immune response have been documented to display unique peculiarities when they derive from cord blood. In fact, cord blood APC produce less interleukin-12 and interleukin-15 than APC of adult peripheral blood[19,20] and, as recently demonstrated,[21] they have characteristics of immaturity, resembling those observed in the so-called type 2 dendritic cells, which are believed to promote tolerance physiologically. Taken together, all these factors can be reasonably supposed to make less efficient both the afferent and efferent phases of the physiopathologic mechanisms of GVHD.

Although UCBT offers a significant advantage over BMT with respect to GVHD occurrence, it is associated with more problems related to hematopoietic reconstitution. In fact, analyses of the patients subjected so far to UCBT have shown that, in comparison with patients given BMT, there is a higher risk of failed engraftment of the donor hematopoiesis, a modest delay in the kinetics of neutrophil recovery, and a more conspicuous delay in platelet reconstitution.[5-12] The engraftment probability and the kinetics of hematologic reconstitution are directly correlated to the number of infused placental cells. Furthermore, the experiences of the EUROCORD group and of the *New York Cord Blood Bank* both unequivocally document that the infused cell load has an important role in the successful outcome of the transplant, since significantly better results have been achieved in subjects who received at least 2-3x10^7 nucleated cells per kg of body weight.[6-10] The better results of transplanted patients receiving a higher number of cells are to be ascribed essentially to a reduction in transplant-related mortality. Since the average content of cord blood nucleated cells per unit before cryopreservation is between 0.8 and 1.5x10^9, it is not surprising that UCBT is usually employed in children with a body weight lower than 35-40 Kg.

In view of the delayed hematopoietic recovery, patients given UCBT should be carefully monitored, in order to detect any onset of bacterial and fungal infectious complications promptly. Likewise, it is considered advisable to perform UCBT in Centers with a solid experience in the treatment of profoundly immunocompromised patients and to administer prophylactic antibiotic and antimycotic agents. For the time being, there is no conclusive evidence that treatment with hematopoietic growth factors active on the granulocytic series (granulocyte colony-stimulating factor or granulocyte-macrophage colony-stimulating factor) can significantly reduce the time required for engraftment.

The reported low incidence of GVHD after UCBT has been hypothesized to be a major drawback in leukemia patients. In fact, since the role of allogeneic lymphocytes in the control and/or eradication of malignancy is well established, the absence or reduction of the component of *graft-versus-leukemia* (GVL) activity associated with GVHD could represent a theoretical concern in leukemia subjects given UCBT. However, available data do not support the hypothesis that patients given UCBT enjoy a lower GVL effect.[9-12]

One previously published study specifically focused on the role of UCBT in children with acute leukemia.[9] Irrespective of whether the cord blood was from a relative or an unrelated donor, the most important factor influencing survival in that cohort was disease state at the time of transplantation. In fact, among all 102 children, those belonging to the good risk group (i.e. children transplanted in 1st or 2nd complete remission) had a significantly better post-transplant outcome than poor-risk patients, 2-year Kaplan-Meier estimates of event-free survival for these groups being 49% and 8%, respectively (p=0.0002). This was a consequence of both an increased one-year transplant-related mortality and a higher 2-year relapse rate in the poor risk group as compared to in the good risk patients. In particular, the 2-year probability of leukemia recurrence in children belonging to the poor- and good-risk groups was 77±14% and 31±9%, respectively (p= 0.01). The impact on relapse rate of disease state at time of transplantation was observed both in patients transplanted from a relative and, even though less evident, in recipients of unrelated UCBT. The increased probability of leukemia recurrence observed in patients given UCBT later in the course of the disease is not surprising given that disease status has been reported to be the most important factor influencing the risk of relapse, irrespective of the source of progenitors (bone marrow, peripheral blood or cord blood) and the type of donor (HLA-compatible relative, partially-matched family donor or unrelated volunteer) employed.[22-25] Even though a formal, well-matched comparison between the relapse rate of UCBT patients and that observed after marrow transplantation from related or unrelated donors was not performed, the outcome of good-risk children appears to be comparable to

those reported in studies on allograft of bone marrow cells from either a family or an unrelated donor in pediatric leukemia patients.[26-28]

These data suggest that UCBT from either a relative or an unrelated donor is a feasible procedure, capable of curing a relevant number of children with acute leukemia, who failed to benefit from conventional chemotherapy or who are at risk of relapse. It is therefore evident that if the aim of UCBT is to achieve a definitive cure in leukemia patients, this transplant should not be considered as a treatment of last resort for patients with end-stage disease, but rather as a well-established and accepted therapy for patients who have achieved a second remission after relapse and for those children who are in first hematologic remission with a high risk of recurrence.

Few data have been published on the biological mechanisms of the UCBT-related GVL effect. In particular, while several authors have demonstrated that cytotoxic T-lymphocytes (CTL) directed against allogeneic leukemia blasts can be detected in the peripheral blood of healthy donors,[29-31] no study on a cord blood T-cell specific HLA-restricted activity towards leukemia blasts has been reported. Innate cell-mediated cytotoxic activities, which are promptly activated by cord blood lymphocytes in response to a primary antigenic challenge,[32] might represent the most important mechanism for controlling the re-growth of leukemia blasts in UCBT recipients. In fact, previously published studies indicate that cord blood lymphokine-activated killer (LAK) cells are able to lyse non-cultured fresh leukemia blasts[15] and that their activity towards cell lines, such as Daudi and YAC-1 cells, is greater than that of bone marrow cells.[33]

In summary, available evidence indicates that for several disorders amenable to cure by an allograft of haematopoietic stem cells, the overall event-free survival with UCBT is not statistically different from that observed after bone marrow transplants. This finding confirms the potential benefit of using umbilical cord blood hematopoietic stem cells for allogeneic transplants.

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References

1. Gluckman E, Broxmeyer HE, Auerbach AD, et al. Hematopoietic reconstitution in a patient with Fanconi anemia by means of umbilical cord blood from a HLA identical sibling. N Engl J Med 1989; 321:1174-8.
2. Rebulla P, Lecchi L Poretti L, et al Practical placental blood banking. Transfus Med Rev 1999; 13:205-26.
3. Armitage S, Warwick R, Fehili, D Navarette C, Contreras M. Cord blood banking in London: the first 1000 collections. Bone Marrow Transplantation 1999; 24: 139-45.
4. Sirchia G, Rebulla P. Placental/umbilical cord blood transplantation. Haematologica 1999; 84:738-47.
5. Wagner JE, Kernan NA, Steinbuch M, Broxmeyer HE, Gluckman E. Allogeneic sibling umbilical cord blood transplantation in forty-four children with malignant and non-malignant disease. Lancet 1995; 346:214-9.
6. Kurtzberg J, Laughlin M, Smith J, et al. Placental blood as a source of hemopoietic stem cells for transplantation into unrelated recipients. N Engl J Med 1996; 335:157-66.
7. Gluckman E, Rocha V, Chammard A, et al. Outcome of cord blood transplantation from related and unrelated donors. Eurocord Transplant Group and the European Blood and Marrow Transplantation Group. N Engl J Med 1997; 337:373-81.
8. Rubinstein P, Carrier C, Scaradavou A, et al. Outcomes among 562 recipients of placental blood transplants from unrelated donor. N Engl J Med 1998; 339: 1565-77.
9. Locatelli F, Rocha V, Chastang C, et al. Factors associated with outcome after cord blood transplantation in children with acute leukemia. Blood 1999; 93: 3662-71.
10. Gluckman E, Rocha V, Chevret S. Related and unrelated cord blood transplantation in Cord blood characteristics role in stem cell transplantation. Edited by SBA Cohen, E. Gluckman, A. Madrigal, and P. Rubinstein. Martin Dunitz editor London pp. 215-6, 2000.
11. Rocha V, Wagner JE, Sobocinski KA, et al. Graft-versus-host disease in children who have received a cord blood or bone marrow transplant from an HLA-identical sibling. N Engl J Med 2000; 342:1846-54.
12. Rocha V, Cornish J, Sievers E, et al. Comparison of the outcome of unrelated bone marrow or cord blood transplant in children with acute leukemia. Blood 1999; 94 (Suppl. 1):712a.
13. Madrigal JA, Cohen SBA, Gluckman E, Charron DJ. Does cord blood transplantation results in lower graft-versus-host disease? It takes more than two to tango. Hum Immunol 1997; 56:1-5.
14. Chalmers I, Janossy G, Contreras M, Navarrete C. Intracellular cytokine profile of cord and adult blood lymphocytes. Blood 1998; 92:11-8.
15. Keever CA, Abu-Hajir M, Graf W, et al. Characterization of the alloreactivity and anti-leukemia reactivity of cord blood mononuclear cells. Bone Marrow Transplant 1995; 15:407-19.
16. Cohen SBA, Perez-Cruz I, Fallen P, et al. Analysis of the cytokine production by cord and adult blood. Hum Immunol 1999; 60:331-6.
17. Risdon G, Gaddy J, Horrie M, et al. Alloantigen priming induces a state of unresponsiveness in human umbilical cord blood T cells. Proc Natl Acad Sci USA 1995; 92:2413-7.
18. Maccario R, Burgio GR. T and NK lymphocyte subpopulations in the neonate. In: Immunology of the neonate. Burgio GR, Hanson L, Ugazio AG, eds. Springer Verlag, Berlin-Heidelberg, 1987. p. 120-9.
19. Lee S, Suen Y, Chang L, et al. Decreased interleukin-12 (IL-12) from activated cord versus adult peripheral blood mononuclear cells and upregulation of interferon-gamma, natural killer and lymphokine-activated killer activity by IL-12 in cord blood mononuclear cells. Blood 1996; 88:945-54.
20. Quian JX, Lee S, Suen Y, et al. Decreased interleukin-

15 from activated cord versus adult peripheral blood mononuclear cells and the effect of interleukin-15 in upregulating anti-tumour immune activity and cytokine production in cord blood. Blood 1997; 90:3106-7.
21. Sorg RV, Kogler G, Wernet P. Identification of cord blood dendritic cells as an immature CD11c- population. Blood 1999; 93: 2302-7.
22. Barrett AJ, Horowitz MM, Gale RP, et al. Marrow transplantation for acute lymphoblastic leukemia: factors affecting relapse and survival. Blood 1989; 74:826-33.
23. Santos GW. Marrow transplantation in acute nonlymphocytic leukemia. Blood 1989; 74:901-14.
24. Gratwohl A, Gahrton G. Allogeneic bone marrow transplantation in leukemia. Acta Oncol 1988; 27: 557.
25. Armitage JO. Bone marrow transplantation. N Engl J Med 1994; 330:827-38.
26. Balduzzi A, Gooley T, Anasetti C, et al. Unrelated donor marrow transplantation in children. Blood 1995; 86:3247-56.
27. Davies SM, Wagner JE, Shu XO, et al. Unrelated donor bone marrow transplantation for children with acute leukemia. J Clin Oncol 1997; 15:557-65.
28. Oakhill A, Pamphilon DH, Potter MN, et al. Unrelated donor bone marrow transplantation for children with relapsed acute lymphoblastic leukaemia in second complete remission. Br J Haematol 1996; 94:574-81.
29. Faber LM, van Luxemburg-Heijs SAP, Willemze R, Falkenburg JHF. Generation of leukemia-reactive cytotoxic T lymphocyte clones from the HLA-identical bone marrow donor of a patient with leukemia. J Exp Med 1992; 176:1283-9.
30. Sosman JA, Oettel KR, Hank JA, Fisch P, Sondel PM. Specific recognition of human leukemic cells by allogeneic T cell lines. Transplantation 1989; 48:486-95.
31. Salomo M, Steinmann J, Glass B, et al. Leukemia-specific allogeneic donor T cells: quantification by limiting dilution assay. Bone Marrow Transplant 1995; 15:179-86.
32. Moretta A, Comoli P, Montagna D, et al. High frequency of Epstein-Barr virus (EBV) lymphoblastoid cell line-reactive lymphocytes in cord blood: evaluation of cytolytic activity and IL-2 production. Clin Exp Immunol 1997; 107:312-8.
33. Gardiner CM, O'Meara A, Reen DJ. Differential cytotoxicity of cord-blood and bone marrow-derived natural killer cells. Blood 1998; 91:207-13.

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Unrelated donor marrow transplantation: an update of the experience of the Italian Bone Marrow Transplant Group (GITMO)

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ABSTRACT

Unrelated donor bone marrow transplant (UD-BMT) has become an attractive alternative source of hematopoietic cells for patients lacking a matched sibling. The aim of this paper was to report on results of the 696 UD BMTs performed in 31 Italian institutions during the first 10 years of activity of the Italian Bone Marrow Donor Registry (IBMDR). In 1989 the Italian Bone Marrow Transplant Group (GITMO) established the IBMDR to facilitate donor search and marrow procurement for patients lacking an HLA identical sibling. By end of December 1999, 260,000 HLA-A, B typed volunteer donors had been cumulatively registered and 2,620 searches had been activated for Italian patients. At least one HLA-A, B, DRB1 matched donor was found for 54% of the patients and 696 UD BMTs were performed. In 50% of cases the donor was found in the IBMDR and in 50% in 15 other Registries. The average time from search activation to transplant was 6 months for disease other than CML. For CML it was 14 months. Actuarial 12-month transplant-related mortality (TRM) was 68% in patients grafted between 1979 and 1992 and 44% for patients grafted after 1993. Twenty-eight per cent of patients developed grade III or IV acute GvHD and 24% developed extensive chronic GvHD. The rate of disease free survival at three years was 57% for patients with 1st chronic phase CML, 37% for patients with 1st or 2nd CR ALL, 31% for AML or MDS patients ≤ 18 years of age and 54% for patients with inborn errors. We conclude that the IBMDR has benefited a substantial number of patients lacking a matched sibling and has facilitated the recruitment of UDs into the international donor pool. The long time required for the search is the major obstacle to the success of this programme. This suggests that early transplant and a decrease in TRM could further improve these encouraging results.

Key words: bone marrow transplant from unrelated donor (UD BMT), Italian Bone Marrow Transplant Group (GITMO), Italian Bone Marrow Donor Registry (IBMDR)

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Allogeneic bone marrow transplantation from HLA-identical siblings has become an effective treatment for patients with haematological malignancies, syndromes of marrow failure, and various inborn errors.[1-3] Since only one out of three patients has a suitable, matched sibling, several investigators have explored the use of alternative sources of haematopoietic stem cells, including HLA-mismatched family members,[4] unrelated donors (MUD),[5] or cord blood (UCBs) units.[6]

The *Italian Bone Marrow Transplant Group* (GITMO) established the *Italian Bone Marrow Donor Registry* (IBMDR) in 1989 to facilitate donor search and marrow procurement for patients lacking an HLA identical sibling. In 1996 we

reported on the initial experience concerning 633 searches activated for Italian patients and 75 BMTs from UDs performed in GITMO institutions.[5] In this report, we present the results of the initial 696 UD BMTs performed in Italy between September 1989 and December 1999.

Design and Methods

IBMDR

The IBMDR was established as a collaborative effort among the Regional Tissue Typing Laboratories. Its principles and policies are similar to those of other international Registries.[7-11] IBMDR includes 17 local Registries and 100 donor centres for the HLA class I and II serological typing of volunteer donors.

Donor recruitment

In December 1992 the number of donors was 25,000. Over the following years more than 20,000 new donors per year have been added to the registry. Currently, the IBMDR file contains data concerning approximately 260,000 volunteers. All of them are HLA-A,B serologically typed, while HLA-DR serological typing has also been performed on 86,000 (33%) of these registered donors. The DRB1 typing by PCR technique of 11,000 donors is also available. Once a month the IBMDR electronically sends its donor HLA phenotype file to the *Bone Marrow Donors World Wide* (BMDWW) directory, which includes more than 6,000,000 donors in its May 2000 edition.[12]

All the Italian transplant centers (TC) involved in this programme are accredited through the GITMO to the IBMDR, to European Registries and to the *National Marrow Program of the United States of America* (NMDP), according to the *World Marrow Donors Association* standards.

Matching criteria

Before December 1991 most TC based their matching criteria on HLA-A,B,DR identity using serologic testing. After January 1992 class II antigens matching was confirmed by DNA techniques as previously described.[5]

Engraftment

Neutrophil engraftment was defined as the first of 3 consecutive days with an absolute neutrophil count (ANC) $>0.5\times10^9$/L, whereas platelet engraftment was defined as the first of 3 consecutive days with an untransfused platelet count $>50\times10^9$/L.

Data collection and statistical analysis

Essential data regarding all donor-recipient pairs, and information concerning the harvesting procedure were obtained by the IBMDR data centre. Information on the BMT procedure and on the recipients follow-up were collected every year by the GITMO Registry. Univariate analysis was performed for relapse, for disease-free survival (DFS), and for transplant-related mortality (TRM). TRM was defined as mortality due to any cause other than disease progression in patients with haematological malignancies. Life tables (Kaplan-Meier estimates) were calculated at 3 years to measure the proportion of patients alive or, for malignancies, alive and disease-free. Data were updated as of December 31st, 1999.

Results

The search

Between September 1st 1988 and December 31st 1999 the search for an UD was started for 2,620 patients. Half of them were activated during the last three years. During the last 12 months, a formal search was started each month for approximately 40 new Italian patients.

Probability of finding a matched donor

At least one HLA-A,B,DRB1 matched donor was found for 54% of the patients. At least one 1 antigen mismatched donor was found for 66% of the patients. At least one HLA-A, B, CW, DRB, DQA, DQB matched donor was found for 28% of the patients.

As of December 1999, 696 Italian patients had undergone UD BMT in one of the 31 TC participating in this program. Three hundred sixty-seven of them were over 18 years of age, while, 329 were 18 years old or less. Half of them were transplanted during the last 25 months. In 1999 each month approximately 15 new Italian patients underwent UD BMT. Details on the activity of the 31 TC are reported in the Appendix. The characteristics of donors and recipients listed according to the type of disease that led to transplant are reported in Table 1.

Donor Registries

The first UD BMT performed in Italy was made possible in March 1989 by a donor listed with the Anthony Nolan Research Foundation. The first UD marrow provided by the IBMDR was grafted in March 1991. By December 1999, 347 donors had been provided by the IBMDR and 349 by other Registries, as shown in Table 2. As of 1997 the IBMDR has become the main source of donors for Italian patients.

Donor Recipient matching

Six hundred out of 696 pairs were HLA-A,B,DRB1 matched, while 80 pairs were mismatched for at least one antigen. Data concerning the remaining 16 pairs are missing. Details on matching by recipient age are reported in Table 3.

Table 1. Characteristics of donors and recipients according to the type of disease that led to transplant.

Disease	*No. pts.*	*Age (years)*				*Gender*			
		donor		*recipient*		*donor*		*recipient*	
		md.	*r.*	*md.*	*r.*	*M*	*F*	*M*	*F*
Neoplastic	601								
CML	251	36	20-58	32	6-52	130	121	169	82
1st chronic phase	145								
2nd chronic phase	74								
Accelerated phase	26								
Blast crisis	6								
ALL	192	35	20-57	14	2-49	100	92	127	65
1st remission	54								
2nd remission	94								
≥3rd remission	44								
AML	80	33	21-55	24	2-51	47	33	48	32
1st remission	30								
2nd remission	34								
≥3rd remission	16								
Myelodysplasia	68	39	22-55	17	1-53	36	31	35	33
Other	10	35	28-45	36	9-49	7	3	4	6
NHL	3								
MM	2								
Non neoplastic	95								
Severe aplasia	12	39	24-51	23	4-43	6	6	4	8
Inborn errors	83	35	20-52	4	2-25	45	38	58	25
Severe combined immunodefic	17								
Non-immunodeficient disorders	66								
TOTAL	696								

CML: Chronic myeloid leukemia; ALL: Acute lymphoblastic leukemia; AML: Acute myeloblastic leukemia; NHL: Non-Hodgkin's lymphoma; MM: Multiple myeloma.md.: median; r.: range; M: male; F: female.

Table 3. Matching by recipient age.

Matching	*No. of pairs by recipient age*	
	≤ 18 yrs	> 18 yrs
A,B,DRB1 =	253	347
1 A locus antigen ≠	23	8
1 B locus antigen ≠	19	4
1 DRB1 antigen ≠	20	1
> 1 antigen ≠	5	-
Unknown	9	7
Total	329	367

Table 4. Conditioning regimens.

	No. of pts
Neoplastic diseases	601
Radiation containing regimens	414
Non-radiation containing regimens	134
Unknown	53
Non neoplastic diseases	95
Radiation containing regimens	21
Non-radiation containing regimens	62
Unknown	12

Table 2. Registries and donors provided to Italian patients.

IBMDR	347
NMDP USA and Canada	102
A. Nolan	87
German	81
French	43
Dutch	10
Swiss	9
Belgian	5
Austrian	2
Spanish	3
Swedish	2
Australian	1
Finnish	1
Norwegian	1
Taiwanese	1

Preparative regimen and graft-versus-host disease (GvHD) prophylaxis

The preparative regimen and GvHD prophylaxis varied depending on the underlying disease, on the transplant centre, and over time. Details are reported in Table 4. Briefly, the preparative regimen for 435 out of 631 patients whose data were available included radiation which was either preceded or followed by one or more drugs. One hundred and ninety six patients were treated with a non-radiation containing regimen. Three hundred and five out of 650 patients, whose data were available, received "serotherapy" (ATG or Campath 1G) containing GvHD prophylaxis; 308 other patients received non-serotherapy containing GvHD prophylaxis, including cyclosporin (CSA) and "short" methotrexate. The remaining 37 patients received other GvHD prophylaxis.

Engraftment

Among 643 evaluable patients, the 30 who died within 3 weeks of transplantation were not evaluated for engraftment. Sustained neutrophil engraftment was reached at a median of 18 days (range 10-48) after transplant by 621 evaluable patients. Sustained platelet engraftment was reached at a median of 27 days (range 11-273) by 390 out of 540 evaluable patients.

GvHD

Two hundred and sixty four of 367 evaluable patients >18 years of age (77%) developed grade I (77, 22%), grade II (94, 27%), grade III (50, 15%), or grade IV (43, 13%) GvHD.

Two hundred and forty four of 329 evaluable patients ≤ 18 years of age (82%) developed grade I (82, 28%), grade II (76, 26%), grade III (51, 17%), or grade IV (35, 11%) GvHD.

Chronic GvHD developed in 148/230 (64%) evaluable patients >18 years of age, and 37 of them (16%) had extensive disease. In patients

Table 5. Timing and causes of transplant related mortality before and after 1992.

	No. of patients			
Year of BMT	*1989-1992*	*37*	*1993-1999*	*659*
Cause of death	*First 100 d After BMT*	*After 100 d From BMT*	*First 100 d After BMT*	*After 100 d From BMT*
GvHD	5	1	51	34
Interstitial pneumonia	2	2	31	13
Infections	4	2	24	15
Unknown			23	10
Graft failure	3	1	6	10
Organ Failure			10	6
Haemorrhage	1		8	3
VOD			10	1
Cardiac Failure	1		5	1
2nd tumour	1	1	2	1
Encephalopathy	1	1	1	2
Other			1	1
Total	18	8	172	97

≤18 years of age it developed in 75/191 (39%) evaluable patients, and 40 of them (21%) had extensive disease.

Transplant-related-mortality (TRM)

Altogether 295 out of 696 patients died of TRM. Between 1989 and 1992 18/37 patients died within the first 100 days after BMT and 8 died after 100 days from BMT. Actuarial 12-month TRM was 68% (C.I. 53-82). Between January 1993 and December 1999, 172/659 patients died within the first 100 days after BMT and 97 patients died after 100 days from BMT. Actuarial 12-month TRM was 44% (C.I. 53-82) (p=001). Timing and causes of TRM are reported in Table 5.

Chronic myeloid leukemia (CML)

Two hundred and fifty one patients with CML underwent UD BMT. The median interval from diagnosis to transplant was 27 months (range 4-119) for 145 patients undergoing BMT in first chronic phase, and 33 months (range 5-130) for 106 patients undergoing BMT in a more advanced phase. The median interval between search activation and transplant was 14 months (range 2-90). Altogether 101 of 251 patients died of TRM. Actuarial 12-month TRM was 38% (CI 33-46).

CML recurrence occurred in 29 patients at a median of 5 months (range 1-59) after BMT. The 3 year relapse rate was 20% (CI 15-30). Three year DFS from time of transplant was 45% (CI 37-51).

Univariate proportional hazard regression analysis was performed for DFS after stratification of the following variables: disease phase, recipient age, HLA matching, conditioning regimen, number of infused cells, donor gender and age, CMV status.

Table 6. DFS of CML patients.

	No. of pts	*DFS %*	*Univariate analysis*
Overall	251		
Disease phase			
total	251	45	
early	145	57	
advanced	106	30	<.001
Recipient age at BMT (years)			
≤ 34	141	51	
> 34	110	38	0.01
LLA matching			
A,B,DRB1 =	229	48	
1 Ag mismatched	17	29	0.01
Other	5		
TBI			
Yes	183	48	
No	53	35	0.07
Unknown	15		
CMV status			
Donor/recipient Neg	26	56	
Donor/recipient Pos	189	44	NS
Unknown	36		
Donor age (years)			
≤ 40	178	48	
> 40	73	38	NS
Infused Cells (x 10^8/kg)			
> 3.5	132	49	
≤ 3.5	106	41	NS
Unknown	13		
Donor gender			
M	130	46	
F	121	44	NS

Univariate analysis showed that early phase at BMT (p <0.001), recipient age <34 years (p = 0.01), and HLA A,B,DRB1 matching (p= 0.01) were associated with higher DFS probability. Other characteristics shown in Table 6 were also associated with higher DFS probability, but the difference did non reach statistical significance, likely due to the low number and heterogeneity of the patients.

Acute lymphoblastic leukemia (ALL)

One hundred and ninety two patients with ALL underwent UD BMT. The median interval between search activation and transplant was 6 months (range 1-27).

Altogether 79/192 patients died of TRM. Actuarial 12-month TRM was 52% (CI 42-58). Leukemia relapse occurred in 38 patients at a median of 6 months (range 2-25) after BMT. Three-year DFS from time of transplant was 33% (CI). In univariate analysis early phase (1st and 2nd CR) at BMT (p = 0.006) was associated with higher DFS probability (37% vs 21%). Other characteristics, including year of transplant and recipient age, made no difference in DFS.

Acute myeloblastic leukemia (AML) and myelodysplasia (MDS)

Sixty patients with AML and 68 with MDS underwent UD BMT. The median interval between search and transplant was 6 months (range 2-31).

Altogether 73/148 patients died of TRM. Actuarial 12-month TRM was 54% (CI 47-65). Disease progression occurred in 30 patients at a median of 4 months after BMT (1-38). Actuarial 3 year relapse rate was 39% CI 30-56).

Three year DFS was 31% (CI 19-43) for 70 patients ≤18 years of age while it was 23% (11-34) for 78 patients >18 years of age (*p*= ns).

Non malignant disorders

Before 1998 only few patients with severe aplastic anemia, mostly in advanced phase underwent UD BMT.

The results of these patients were disappointing. However, preliminary data of a prospective multicentric trial designed for patients in an earlier phase seem encouraging.

Eighty three patients with inborn errors underwent UD BMT. Underlying diagnosis is reported in Table 7. The median interval between search activation and transplant for these patients was 6 months (range 1-38). Altogether 27 out of 83 patients died of TRM. Actuarial 12-month TRM was 30% (CI 21-42). Graft failure and recurrence of the underlying disease occurred in 13 patients at a median of 1 month (1-12) after BMT. Three year DFS was 54% (CI).

Table 7. Diagnosis of 83 patients with inborn errors undergoing UD BMT.

Diagnosis	*No. of pts*	*ADF*	*AWD*	*Dead*
SCID	19	15	-	4
Thalassemia	18	10	4	4
Fanconi anaemia	13	3	-	10
Storage disorders	9	7	-	2
HLH	8	4	-	4
Wiskott Aldrich S.	8	6	-	2
Osteopetrosis	4	2	1	1
Shwachman S.	1	1	-	-
Chediak Higashi	1	1	-	-
Kostman	1	-	-	1
Dyserythropoietic anaemia	1	-	-	1
Total	83	49	5	29

Abbreviations: ADF=alive disease free; AWD=alive with disease; SCID=severe immunodeficiency; HLH=hemolymphohistiocytosis

Discussion

The reported experience is encouraging and shows that a great deal of people are, indeed, willing to donate their bone marrow in Italy and around the world. This has been made possible thanks to good cooperation between volunteers and medical and non-medical staff.

Our data show that an acceptable donor was found for 66% of the patients. This is approximately 50% higher than what we reported in 1996.[5] The recruitment of more than 100,000 Italian donors over the last 36 months will probably further increase the number of patients who will find an UD in the IBMDR.

In our study the average time from search activation to transplant was 6 months for diseases other than CML. In CML the median interval between search activation and transplant was 14 months. Actuarial 12-month TRM was 68% in patients grafted between 1979 and 1992 and 44% for patients grafted between January 1993 and December 1999. The long amount of time required for the search, and the salvage chemotherapy before transplant are likely the main reasons for the high TRM rate in patients with acute leukemia. This suggests that early transplant and a decrease in early TRM could substantially change the outcome of these patients.

Most reports claim that disease stage and chronic phase duration of CML patients are major determinants of outcome.[13,14] In the present report very few patients received transplant within one year of diagnosis and 106 out of 251 were no longer in first chronic phase at the time of BMT. The high risk of TRM as well the chance of severe GvHD were causes of concern for UD BMT early in the course of the disease. Moreover, most patients were part of a prospective study aimed at assessing the efficacy of 1 year IFN-α administration.

It must be noted that every year 15-20% of patients progress to blastic crisis and are denied the transplant option. Moreover, the decreased risk of TRM observed in patients grafted after 1992 produced a significant improvement in prognosis of patients receiving BMT from an "extensively" matched UD after a TBI-containing regimen.[15] This is why the search for an UD for CML patients below 45 years of age should be activated at diagnosis. An initial trial of IFN-a may be appropriate for these patients as well as for those over 45 years old. Allogeneic transplant early in the course of disease may be the approach of choice for patients below 45 years of age with an available donor.

The results we obtained in patients with inborn errors are similar to other series[16-19] and confirm our previous reports.[20,21] SCID, HLH and Wiskott-Aldrich's disease represent an absolute indication to UD BMT. TRM is the major obstacle to the success of this procedure in patients with Fanconi's anemia and osteopetrosis. The best conditioning regimen for this disease is still to be identified. Long-term follow-up

of children with storage disorders suggests that after BMT the loss of intelligence continues despite persistent engraftment and normalisation of enzymatic activity. Therefore, early diagnosis is very important if an UD BMT is to be attempted.[22]

In a previous report we showed that rejection was the main problem for thalassemic patients who undergo UD BMT.[21] The incidence of rejection seems to have decreased with the introduction of thiotepa in the conditioning regimen of more recent patients (data not shown). Indication for UD BMT is still a crucial problem in the therapeutic decision for thalassemic patients lacking a matched sibling. In these cases extensive DNA study of class II antigens is recommended and the patient should proceed to transplant only if a fully matched donor is available.

References

1. Thomas ED. Karnofsky memorial lecture: marrow transplantation for malignant disease. J Clin Oncol 1983; 1:517-31.
2. Champlin RE, Gale RP. The role of bone marrow transplantation in the treatment of hematologic malignancies and solid tumors: a critical review of syngeneic autologous and allogeneic transplant. Cancer Treat Rep 1984; 68:145-61.
3. Fischer A, Landais P, Friedrich W, et al. Bone marrow transplantation in Europe for primary immunodeficiency: a report from the European Group for BMT and the European Group for Immunodeficiency. Blood 1994; 83:1149-54.
4. Aversa F, Tabilio A, Velardi A, et al. Treatment of high-risk acute leukemia with T-cell-depleted stem cells from related donors with one fully mismatched HLA haplotype. N Engl J Med 1998; 339:1186-93.
5. Dini G, Lanino E, Lamparelli T, et al. Unrelated donor marrow transplantation: initial experience of the Italian Bone Marrow Transplant Group (GITMO). Bone Marrow Transplant 1996; 17:55-62.
6. Locatelli F, Rocha V, Chastang C, et al. Factors associated with outcome after cord blood transplantation in children with acute leukemia. Eurocord-Cord Blood Transplant Group. Blood 1999; 93:3662-71.
7. Oudshoorn M, Cornelissen JJ, Fibbe WE, et al. Problems and possible solutions in finding an unrelated bone marrow donor. Results of consecutive searches for 240 Dutch patients. Bone Marrow Transplant 1997; 20:1011-7.
8. McCullough J, Rogers G, Dahl R, et al. Development and operation of a program to obtain volunteer bone marrow donors unrelated to the patient. Transfusion 1986; 26:315-23.
9. McElligott MC, Menitove JE, Aster RH. Recruitment of unrelated persons as bone marrow donors. A preliminary experience. Transfusion 1986; 26:309-14.
10. Darke C. An overview of the Welsh Bone Marrow Donor Registry: 10 years of bone marrow donor provision. Bone Marrow Transplant 2000; 25:771-7.
11. Kodera Y, Morishima Y, Kato S, et al. Analysis of 500 bone marrow transplants from unrelated donors (UD-BMT) facilitated by the Japan Marrow Donor Program: confirmation of UR-BMT as a standard therapy for patients with leukemia and aplastic anemia. Bone Marrow Transplant 1999; 24:995-1003.
12. Bone Marrow Donors World Wide, May 2000 Edition
13. Thomas ED, Clift RA, Fefer A, et al. Marrow transplantation for the treatment of chronic myelogenous leukemia. Ann Intern Med 1986; 104:155-63.
14. Goldman JM, Szydlo R, Horowitz MM, et al. Choice of pretransplant treatment and timing of transplants for chronic myelogenous leukemia in chronic phase. Blood 1993; 82:2235-8.
15. Dini G, Lamparelli T, Rondelli R, et al. Unrelated donor marrow transplantation for chronic myelogenous leukaemia. Br J Haematol 1998; 102:544-52.
16. Kernan NA, Bartsch G, Ash RC, et al. Analysis of 462 transplantations from unrelated donors facilitated by the National Marrow Donor Program. N Engl J Med 1993; 328:593-602.
17. Cornish J. Unrelated Donor Bone Marrow Transplantation in children. In: Steward CG, Hobbs JR eds. Correction of genetic disease by transplantation III, Middlesex GB The COGENT press, 1995:109.
18. Krivit W., Shapiro E.: Marrow Transplantation for storage diseases: the plan and need for a control study. In: Forman S, Thomas ED, Blume K eds. Bone marrow transplantation. Cambridge, MA. Blackwell 1993:883.
19. Gerritsen EJ, Vossen JM, Fasth A, et al. Bone marrow transplantation for autosomal recessive osteopetrosis. A report from the Working Party on Inborn Errors of the European Bone Marrow Transplantation Group. J. Pediatr 1994; 125:896-902.
20. Dini G, Miano M., Mazzolari E, et al. Unrelated donor marrow transplantation for genetic diseases. Bone Marrow Transplant 1997;19(Suppl.2):176-82.
21. Miano M, Porta F, Locatelli F, et al. Unrelated donor marrow transplantation for inborn errors. Bone Marrow Transplant 1998; 21(Suppl. 2):37-41.
22. Miano M, Lanino E, Gatti R, et al. Four years follow up of a case of fucosidosis treated with unrelated donor bone marrow transplantation. Bone Marrow Transplant 2000 (in press).

APPENDIX

Transplant Centers. Number of unrelated donor transplants by year and principal investigator.

CIC Transplant Centre	No. of BMTs ≤1994	≥1995	Total	Main investigators
• *Dipartimento di Ematologia, Ospedale S.Martino - Genova*	21	98	119	A. Bacigalupo, T. Lamparelli
• *Clinica Pediatrica, IRCCS Policlinico S. Matteo - Pavia*	20	57	77	F. Locatelli, G. Giorgiani
• *U.O. Ematologia-Oncologia Pediatrica, IRCCS G.Gaslini - Genova*	14	39	53	G. Dini, E. Lanino
• *Unità TMO, Dipartimento di Ematologia, Ospedale Careggi, Firenze*	4	44	48	A. Bosi, S. Guidi
• *Istituto di Ematologia e Oncologia Medica Seragnoli, Ospedale S.Orsola, Università di Bologna*	5	39	44	G. Bandini
• *Dipartimento di Ematologia, Unità TMO, Policlinico S. Matteo, Pavia*	8	33	41	E.P. Alessandrino
• *Clinica Pediatrica, Università di Brescia*	10	25	35	F. Porta, E. Mazzolari
• *Clinica Pediatrica, Ospedale S. Gerardo, Monza*	4	22	26	C. Uderzo, A. Balduzzi
• *Clinica Pediatrica e Centro Leucemie Infantili, Università di Padova*	3	23	26	C. Messina, S. Cesaro
• *Dipartimento di Pediatria, Ospedale Regina Margherita, Univ. di Torino*	8	16	24	F. Fagioli, E. Vassallo
• *Dipartimento di Biotecnologie Cellulari e Ematologia, la Sapienza, Roma*	-	23	23	W. Arcese, A. Iori
• *Dipartimento di Ematologia, Ospedale Niguarda Ca' Granda, Milano*	5	15	20	P. Marenco
• *Istituto di Ematologia, Università di Udine*	-	18	18	R. Fanin
• *Centro TMO, U.O. Ematologia, Azienda Ospedaliera S. Giovanni Torino*	-	16	16	M. Falda, F. Locatelli
• *Unità TMO, IRCCS Ospedale Maggiore, Milano*	4	11	15	D. Soligo, E. Tagliaferri
• *Dipartimento di Scienze Mediche e Genetica Medica, Università di Cagliari*	3	11	14	G. La Nasa
• *Dipartimento di Ematologia e Centro Trapianti, Ospedale di Pesaro*	14	14		C. Giardini
• *Unità TMO, Clinica Pediatrica, Università di Bologna*	13	13		A. Pession
• *Divisione di Ematologia e Unità TMO, Ospedale V. Cervello, Palermo*	13	13		R. Scimè
• *Dipartimento di Ematologia, Ospedale Civile, Pescara*	3	9	12	P. Di Bartolomeo
• *Divisione Ematologia, Ospedale di Bergamo*	10	10		T. Barbui, A. Rambaldi
• *Dipartimento di Ematologia, Ospedale S. Bortolo, Vicenza*	10	10		F. Rodeghiero
• *Clinica Pediatrica – Centro Trapianti Ospedale Burlo Garofolo, Trieste*	2	4	6	M. Andolina, M. Rabusin
• *Ematologia - Policlinico Borgo Roma, Università di Verona*	5	5		F. Benedetti
• *Centro Trapianti, 2° Clinica Pediatrica, Università di Cagliari*	3	3		F. Argiolu
• *Dipartimento di Ematologia, Policlinico Monteluce, Università di Perugia*	3	-	3	F. Aversa
• *Istituto di Clinica Pediatrica, Università di Pisa*	2	2		C. Favre
• *Azienda Ospedaliera Bianchi-Melacrino- Morelli, Reggio Calabria*	2	2		P. Iacopino
• *Istituto Semeiotica Medica, Ematologia, Università Cattolica Sacro Cuore, Roma*	2	2		G. Leone
• *Divisione di Ematologia, Università di Napoli*	1	1		B. Rotoli
• *Divisione di Ematologia, Ospedale S. Camillo, Roma*	1	1		A. De Laurenzi

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Haploidentical bone marrow transplantation in leukemia and genetic diseases

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ABSTRACT

From 1986 to June 2000, sixty children suffering from acute and chronic leukemia (n = 42, 33 of which in resistant relapse), genetic diseases (n = 11), aplastic anemia (n = 2, one of which with platelet refractoriness and bleeding), myelodysplasia (n = 5) received an haploidentical bone marrow, mismatched for 2-3 HLA loci. The donor's marrow was treated in vitro with vincristine and methylprednisolone to obtain a functional T depletion (MLC and CTL inhibition, functional blockade of Th1 and Th2). The prevalence of infectious complications and GVHD was similar to that recorded in matched unrelated donor (MUD) transplants. In situations of high risk of rejection (chronic leukemia, genetic diseases) we infused immediately one half of the harvest and then frozen aliquots from the second week. Of the 25 ALL and 8 AML in resistant relapse, 3 survived, disease-free at 14, 8 and 1 years respectively. Of the 3 ALL, transplanted during remission, 1 is surviving at 18 months. Of the 6 CML, 1 had fractionated bone marrow and is surviving at 3 years, and 5 had standard single dose infusion and died of progression of their disease after rejection of the graft (4) or blast crisis after complete engraftment (1). The 2 patients with aplastic anemia, those with myelodysplasia, and 6 of the 10 with genetic disorders died of transplant-related complications or disease progression. 4 patients with osteopetrosis (n=2), MLD (n=1), Wiskott Aldrich dis. (n=1) survive at 8, 2, 5 and 1.5 years respectively. In patients transplanted with fractionated marrow GVHD >2nd grade occurred in 15%. Only one patient rejected the graft. Compared with MUD transplantation, mismatched BMT whenever performed in patients in good conditions provides similar outcome and widens the donor availability.

Key words: haploidentical, mismatched bone marrow transplantation, leukemia, genetic diseases

About 40 percent of patients fail to find a perfectly matched donor among relatives on in any of the donor registries.[1] Therefore, more than one third of the patients who could be theoretically cured by a bone marrow transplantion fails to exploit this treatment.[1,2] It has been shown that transplants using mismatched bone marrow can be as effective as those in which the donor and recipient are fully matched.[7,8] Mismatched bone marrow transplant increases the chances of finding a donor and shortens the time for donor search. In fact, the timing of the transplant is critical especially in patients with ALL in CR2 and genetic diseases for whom the BMT is a urgent procedure.[1,2,11]

Haploidentical siblings and parents are suitable donors, whenever healthy and of a proper age and body size. Even if the first experiences of 2-3 loci mismatched transplants were performed more than 15 years ago,[3] this procedure did not obtain a large approval from most centres. However there is an increasing evidence that a mismatched BMT is able to overcome the most severe clinical situations.[4-6] Among strategies of performing the 2-3 loci mismatch transplant and not to perform the transplant when an HLA identical donor is not available, we adopted the former in 1986. Since then, we have performed 60 transplants and the follow up data are similar to those of MUD, with the advantage that any patient is given opportunity for treatment.

Selection of the patients

In the first series of patients we treated mainly children with resistant relapse of acute leukemia (25). These patients had not only resistant blasts in their marrow and peripheral blood, but in most cases suffered by several untoward effects of chemotherapy (infections, liver damage).

Later we began transplanting even chronic leukemias (6), acute leukemias in remission beyond the first (3), genetic diseases (13), severe aplastic anemia (2) and myelodysplastic syndromes(5). Since 1996, the patients who had not previously undergone heavy treatment with immunosuppressive drugs, and therefore were particularly susceptible to rejecting the marrow, received a conditioning regimen (protocol #2) aimed at reducing this danger.

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Treatment of donor marrow

The donor's marrow was harvested in general or epidural anesthesia. The nucleated cells were concentrated to 100 mL by sedimentation and centrifugation.

This cell suspension was incubated at 38.5°C with Vincristine 1.5 µg/mL and Methylprednisolone 3 mg/mL.

The cells were then quickly infused (within 15 minutes) without washing the drugs, or cryopreserved in liquid nitrogen in 4.5 mL tubes.

Conditioning regimen and GVHD prophylaxis

The regimens were different in the different periods according to the various diseases.

In acute leukemias, the risk of rejection is low and the protocols were based mainly on total body irradiation (TBI) or busulphan, and cyclophosphamide, antilymphocyte globulin (ALG) or antithymocyte gobulin (ATG) plus a fourth drug.

Protocol #1

TBI: 750 cGy at high dose rate (20 cGy/m^2); 1200 cGy fractionated in six doses or Busulphan 480 mg/m^2 over 4 days.

Cyclophosphamide (Cy): 3600 mg/m^2 in 2 doses; 6000 mg/m^2 in 4 doses.

ALG-ATG: 5 vials/m^2/day 12 hours after the first dose of Cy in continuous infusion until the night before the marrow infusion (4-12 hours before). Therefore, the number of days of treatment varied from 3 to 5.

Fourth drug: from 1986 to 1992 this was vincristine 4 mg/m^2 in continuous infusion over 4 days; from 1993 this is Thiotepa 300 mg/m^2.

Protocol #2

Conditioning regimen for situations at high risk of rejection.

This protocol is based on busulphan, Cy, thiotepa plus a long-lasting infusion of ALG that begins 12 hours after the first dose of CY and lasts until day +6.

The donor's marrow is divided into two parts: the first one is treated *in vitro* with VCR-MP and immediately infused into the recipient, the second one is treated with the drugs, divided in approximately 20 vials and frozen in liquid nitrogen. The frozen vials are infused (one vial = 0.1x 10^7/kg) on days + 7, 8, 9, 10, 22, 23, and 100.

Results

Engraftment and GVHD

Protocol #1 succeeded in engrafting in 32 out of 33 patients with acute leukemias, while it failed to obtain engraftment in 4 cases of CML out of 5, 2 SAA out of 2, and 5 MDS out of 5. The incidence of GVHD was 65%, while that of GVHD worse than grade 2 was 24%.

Protocol #2 was used in one patient with CML (a long survivor with full engraftment), and in 10 cases of genetic diseases (9 engraftments out of 10). Two patients with osteopetrosis survived for more than one year disease free, as did a patient with Wiskott-Aldrich syndrome and a patient with metachromatic leukodystrophy.

The incidence of GVHD was 30% while that of GVHD worse than grade 2 was 15%.

Table 1.

Cases	*Protocol (number of patients)*
ALL in resistant relapse (25)	1
AML in relapse (8)	1
ALL in CR2 (2)	1
ALL in CR1 (1)	1
Chronic myeloid leukemia (6)	1 (5), 2 (1)
Refractory anemia with excess of blasts (5)	1
Severe aplastic anemia (2)	1 (1), 2 (1)
Ostepetrosis (4)	2
Metachromatic leukodystrophy(2)	2
Adrenoleukodystrophy (1)	2
Krabbe's disease (1)	1
Omenn's disease (1)	1
Chronic granulomatous disease (1)	1

ALL=acute lymphocytic leukemia; CR2=2nd complete remission; CR1=1st complete remission.

Survival

The overall survival was very poor in acute leukemias: only three patients survive disease-free. One patient transplanted in fifth relapse is still alive after 14 years. The main causes of death were early toxicity and relapse.

The survival of patients with CML was poor when transplanted with protocol #1 due to rejection, autologous engraftment and subsequent progression of disease (2 cases), blast crisis (first testicular and then marrow relapse) after complete engraftment (1 case), late viral infection (1 case). One patient transplanted with protocol #2 is alive and well after 2 years.

Most patients with genetic diseases, but for the first 3 (chronic granulomatous disease, Krabbe's disease and Omenn's disease) who did not survive, were transplanted with protocol #2. Four out of ten are alive, disease-free and without chronic GVHD.

Two of the four patients with osteopetrosis alive after 1-8 years (protocol #2). One patient died of a transplant-related cause (infection in the second month) after full engraftment and one patient died one year after BMT of hemolytic anemia (donor's lymphocytes against donor's red blood cells) not transfused according to the

parents wishes (the patient was blind).

One patient with metachromatic leukodystrophy is a long-term survivor (5 years), another one died of progression of disease after one year (full engraftment). One case with adrenoleukodystrophy had an uneventful follow-up but died of disease progression.

A patient with Wiskott-Aldrich disease survives more than one year after transplantation, and has mild signs of chronic GVHD.

Discussion

Our protocol of conditioning and mainly of treating the donor's marrow allows us to perform mismatched transplants against the HLA barrier. The complications related to the HLA mismatch (GVHD, rejection) were not worse than those occurring after transplants from matched unrelated donors (MUD). Our donor's marrow treatment is certainly the cheapest, and provides blockade of MLC, Th1 and Th2 functions without inducing apoptosis.[9] In our opinion, compared to methods that kill or separate T-lymphocytes, it offers a lower risk of long-lasting immune deficiency.

Patients with acute leukemias resulted to very easy to engraft since their immune systems were already damaged by previous heavy chemotherapy. The engraftment was more difficult in diseases not previously heavily treated (CML and genetic diseases) until we decided to use protocol #2 that provides a selective advantage to the donor's marrow. In this protocol the infusion of ALG before and after the infusion of the marrow achieves an *in vivo* T-depletion of both the recipient's and the donor's blood. After the first week from BMT. Infusions of small aliquots of donor's marrow reconstitute only the donor's immune system (without increased risk of GVHD) and enlarge the patient's myeloid stem cell pool.

Osteopetrosis is probably the disease in which the engraftment of a mismached marrow is the most difficult; most of the haploidentical transplants performed so far failed due to rejection.[10,11] In our experience four engraftments out of four is not a minor result. Only one patient could be considered a failure related to the procedure (late viral infection); a second death occurred one year later due to an autoimmune hemolytic anemia (lymphocytes and red blood cells were of donor's origin) and the refusal of the parents to transfuse the completely blind child.

The availability of an HLA compatible donor within a family is lower in genetic diseases than in leukemia, and in this case the use of a haploidentical donor could be considered. In metachromatic leukodystrophy (MD), as others, we witnessed a progression of the disease after BMT, but in one patient we were able to demonstrate a clear improvement of spasticity and attention to the environment after the intrathecal injection of irradiated donor's leukocytes, as if the cells could have baypassed the blood brain barrier. Two other patients with metachromatic leukodystrophy not submitted to BMT improved a few weeks after the same intrathecal treatment. The effect lasted two weeks, and was reproduced soon after every further injection.

In our opinion a BMT should be offered to children with MLD if associated with intrathecal injections at least in the first six months after the transplant; it is hoped that later the marrow donor's cells should slowly pass through the blood-brain barrier.

As compared to our limited experience with MUD transplants a 3 loci mismatched BMT is easier to perform. We must bear in mind that a parent can be used as a donor in any time, therefore allowing us to choose the right moment every time. Whenever damage to the donor's marrow is unavoidable (viral infection, drugs, rejection) there is no problem in obtaining a second harvest.

The major advantage of a haploidentical BMT over a MUD BMT is definitely the possibility of choosing the right time for the transplant. This is particularly true in the second remission of ALL, in which the timing is essential. All too often we witness the sad situation in which physicians chose to wait until another last relapse while looking for a matched unrelated donor. It is well known that half of the patients experience a second relapse within six months of the first one. The same situation occurs in other diseases, for example osteopetrosis and adrenoleukodystrophy. Two infants were blind at the time of the transplant, and a patient with adrenoleukodystrophy had an hopeless progression even though we lost no more than one month from the time of the first contact with the patients.

We think that in every disease we must be aware of how much time we may wait for the search of a compatible donor. The management of each patient should have its own timing; after the maximum bearable time a patient must be submitted to "a transplant", and a haploidentical BMT should be considered as a reasonable choice.

References

1. Pession A, Rondelli R, Paolucci P, et al. Hematopoietic stem cell transplantation in childhood: report from the bone marrow transplantation group of the Associazione Italiana Ematologia Oncologia Pediatrica (AIEOP). Haematologica 2000; 85:638-46.
2. Dini G, Miano M, Morreale G, Lanino E. Transplan-

tation of bone marrow from unrelated donors in Italy: activity and results. Ann Ist Super Sanita. 1999; 35:7-11.
3. de Manzini A, Andolina M, Agosti E, et al. Low incidence of GvHD and rejection after pharmacological ex vivo modulation of bone marrow in 2-3 antigens mismatched BMT. Bone Marrow Transplant 1993;11 Suppl 1:114-6.
4. Bunjes D, Duncker C, Wiesneth M, et al. CD34+ selected cells in mismatched stem cell transplantation: a single centre experience of haploidentical peripheral blood stem cell transplantation. Bone Marrow Transplant 2000; 25 Suppl 2:S9-11.
5. Goldman FD, Rumelhart SL, DeAlacron P, et al. Poor outcome in children with refractory/relapsed leukemia undergoing bone marrow transplantation with mismatched family member donors. Bone Marrow Transplant 2000; 25:943-8.
6. Godder KT, Hazlett LJ, Abhyankar SH, et al. Partially mismatched related-donor bone marrow transplantation for pediatric patients with acute leukemia: younger donors and absence of peripheral blasts improve outcome. J Clin Oncol 2000; 18:1856-66.
7. Aversa F, Terenzi A, Carotti A, et al. Improved outcome with T-cell-depleted bone marrow transplantation for acute leukemia. J Clin Oncol 1999; 17:1545-50.
8. Aversa F, Terenzi A, Felicini R, et al. Mismatched T cell-depleted hematopoietic stem cell transplantation for children with high-risk acute leukemia. Bone Marrow Transplant 1998; 22(Suppl 5):S29-32.
9. Lanfranchi A, Andolina M, Tettoni K, et al. Functional depletion of T cells by vincristine and methylprednisolone as an in vitro model for the prevention of graft versus host disease. Haematologica 1992. 77:11-5.
10. Fasth A, Porras O. Human malignant osteopetrosis: pathophysiology, management and the role of bone marrow transplantation. Pediatr Transplant 1999; 3 (Suppl 1):102-7.
11. Eapen M, Davies SM, Ramsay NK, Orchard PJ. Hematopoietic stem cell transplantation for infantile osteopetrosis. Bone Marrow Transplant 1998; 22: 941-6.

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Haploidentical peripheral blood and marrow stem cell transplantation in nine cases of primary immunodeficiency

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ABSTRACT

Bone marrow transplantation (BMT) is the treatment of choice in children affected by primary immunodeficiency (PID). Because only 10-15% of affected children have a familial HLA-identical donor alternative therapeutic options are BMT from a matched unrelated donor or an haploidentical BMT. In our experience only 40% of these children find a donor within the International Registry. Therefore, the remaining 50% children affected by PID are candidates for haploidentical BMT. Unfortunately, in PID other than sever-combined immunodeficiency (SCID), low engraftment rates have been reported because of minimal residual immunity. In order to enhance engraftment rate in haploidentical BMT in PID we suggest a protocol with addition of donor peripheral stem cells after mobilization with granulocyte colony-stimulating factor (G-CSF) (16 µg/kg for 5 days) and bone marrow cells. This procedure increases the cell load, which allows intensification of the conditioning regimen for induction of faster engraftment. The separation of $CD34^+$ cells from leukapheresis products was achieved in the first 6 patients by the Isolex 300 system (Baxter) with a $CD34^+$ cell purity range of 80-95% and in another three patients by the Clinimacs System (Miltenyi). The peripheral blood stem cells were cryopreserved until BMT, 15 days after G-CSF stimulation when the bone marrow was harvested, processed and T-cell depleted with Campath 1-M in the first 6 cases while the Clinimacs System was used in the remaining cases and no T-cell depletion was required. We included 9 patients in the study protocol: SCID (4), Omenn's syndrome (3), LAD (1) and CID (1). The mean value of peripheral $CD34^+$ cells infused was 13.42×10^6/kg and the mean $CD3^+$ cells number was 0.385×10^5/kg; the mean value of BM $CD34^+$ cells infused was 10.62×10^6/kg and the mean $CD3^+$ cell number was 2.39×10^5/kg.The mean number of infused CFU was 8.1×10^5/kg for PBSC and 3.59×10^5/kg for BM. The 9 patients achieved more than 0.5×10^9 peripheral blood neutrophils/L at a mean of 14.6 days (range: 6-22 days). One patient affected by SCID showed complete chimerism, but he died after BMT of systemic CMV infection; the other 8 patients are alive and well and 4 of them show complete chimerism in all cell lines. Split chimerism was documented in 2 SCID cases ($CD3^+$ lymphocytes were of donor origin, monocytes were autologous and granulocytes were mainly autologous); 1 patient affected by Omenn's syndrome received 3 transplants (1 from the mother and 2 from the father, T-cells alone and bone marrow) and achieved engraftment with complete chimerism after the third transplant; the patient affected by LAD also received 3 transplants (2 bone marrow infusions and 1 PBSC infusion) achieving complete chimerism after the third one. In conclusion, the engraftment achieved in all treated patients, and the acceptable conditioning-related toxicity suggest that this approach could be successfully applied to children affected by PID and candidates for haploidentical BMT.

Key words: bone marrow transplantation, primary immunodeficiency , peripheral blood stem cells

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Primary immunodeficiencies (PID) are a heterogeneous group of congenital diseases that affect cellular and/or humoral immunity, resulting in abnormal susceptibility to infections.[1] The combined defects of the two immune compartments (severe combined immunodeficiencies, SCID; combined immunodeficiencies, CID) lead to severe infections and are usually fatal within the first year of life. Bone marrow transplantation (BMT) is the only treatment option for patients affected by SCID.[2] Only about 10-15% of these children have a family matched donor, so that an alternative donor is required. Almost half of the patients who lack a matched family donor can be successfully treated with BMT from an HLA-matched unrelated donor found within the *International Bone Marrow Donor Registry*[3] The remaining patients require a haploidentical BMT. The results of haploidentical BMT are good (75% overall survival) only in patients affected by SCID with B-cells (SCID T-B^+). For other diseases such as SCID T-B-, Omenn's syndrome, Wiskott-Aldrich syndrome,

phagocytic cell diseases, hemophagocytic lymphohistiocytosis or CID the overall survival rate is about 40-50%, because of the high incidence of graft failure, delayed immunologic reconstitution and graft-versus-host disease (GVHD).[4,5] The conventional conditioning regimen for primary immunodeficiences consists of busulfan (4 mg/kg/day for four days) and cyclophosphamide (50 mg/kg/day for four days). This protocol is effective, but is probably insufficiently myeloablative and immunosuppressive to establish tolerance of the graft in patients with PID. In fact, these procedures allow some cells of host origin to remain and those can then be responsible for graft failure. There is also evidence of competition between donor and residual host stem cells for the limited available niches in the BM stroma as well as the availability of facilitating cells in the donor stem cell source.[6-8] New strategies have, therefore, been suggested in order to overcome these problems. In this clinical setting our approach is to intensify the conditioning regimen and to increase the stem cell inoculum. We designed a conditioning regimen protocol with addition of thiotepa, a powerful myeloablative agent, to the conventional busulfan and cyclophosphamide regimen, in order to enhance both immunosuppression and myeloablation.[9] At the same time we attempted to increase the overall number of $CD34^+$ cells infused by adding positively selected peripheral blood $CD34^+$ cells to T-depleted bone marrow aiming to increase engraftment rate in PID with residual cellular immunity (Omenn's syndrome, Wiskott-Aldrich syndrome, SCID with maternal engraftment, SCID T-B-NK+, CID, phagocytic diseases).

Design and Methods

Patients

We enrolled 9 patients (5 males/4 females) in the study. These patients were candidates for haploidentical BMT, affected with PID at an increased risk of graft failure: SCID with maternal engraftment (4), Omenn's syndrome (3), CID (1) and LAD (1). The range of their ages at BMT was 4-18 months (mean, 10.3 months). Haploidentical donors were the mother in 2 patients, the father in 4 patients, an aunt, identical to the mother, in 1 case; 2 patients received multiple transplants (1 from the mother and 2 from the father; 3 transplants from the mother).

Conditioning regimen

The conditioning regimen consisted of busulfan (4 mg/kg/d for 4 days from day -9 to day-6) and cyclophosphamide (50 mg/kg/d for 4 days from day –5 to day –2); all patients received thiotepa intravenously (10 mg/kg) on day-4.

Supportive care

Patients were cared for in laminar air-flow rooms until the leukocyte count recovered to more than 200/mm^3. All patients received prophylaxis for *Pneumocystis carinii* (trimethoprim-sulfamethoxazole), paromomicin for gut decontamination, fluconazole, intravenous immunoglobulins (400-600 mg/kg/2-4 weeks) and total parenteral nutrition. Fever that occurred in the period of neutropenia was treated with broad-spectrum antibiotics. Cytomegalovirus (CMV) prophylaxis consisted of gancyclovir. Foscarnet was used when CMV infection or re-activation was documented by direct immunofluorescence. Patients were supported with filtered and irradiated blood products.

GVHD prophylaxis and treatment

Six patients received cyclosporin A orally from day -1 for GVHD prevention; in the remaining cases no GVHD prophylaxis was applied. Two patients received antithymocyte gammaglobulin (ATG) before the BMT and three patients received methylprednisolone at the onset of acute GVHD.

Engraftment and immunologicstudies

Engraftment was defined as the achievement of $0.5x10^9$ neutrophils/L. Chimerism was analyzed by restriction fragment length polymorphism (RFLP)[10] in peripheral blood cells. Lymphocyte subpopulations were analyzed by two-color immunofluorescence and flow cytometry. T-cell function was assessed by stimulating cells with mitogens according to standard protocols.

Bone marrow and peripheral blood mononuclear cell collection

Donor bone marrow (BM) cells were obtained under general anesthesia by multiple aspirations from both iliac crests. Mobilization of donor peripheral blood stem cells (PBSC) was obtained by administering rhG-CSF subcutaneously (16 µg/kg daily for 5 days); the PBSC were then collected by leukapheresis.

BM and PBSC processing

In the first 6 cases and the first transplant of the 7th case BM suspensions were depleted of T-lymphocytes by monoclonal antibody Campath 1M,[11] while all PBSC but one, after CD34 positive selection, were T-cell depleted by the E-rosetting technique.[12] One patient received BM and PBSC T-cell depleted exclusively by Campath 1M. Positive selection of PBSC $CD34^+$ cells was performed by the ISOLEX 300 selection system.[13] The cell suspension was incubated with the murine anti-human CD34 monoclonal antibody. The unbound antibody was then removed by

washing the cell suspension and the positive cells rosetted with the Dynal paramagnetic microspheres, coated with sheep anti-murine antibody. The $CD34^+$ cells were magnetically separated from the cell suspension using the ISOLEX 300 selection separator. Negative cells were removed by washing and target cells were separated from immunomagnetic beads by cleaving the protein linkage by chimopapain (Chymocell-T). The colony-forming units (CFU) of granulocytes and macrophages (GM) present in BM and PBSC were measured by plating cells (10^4 cells/plate) in methylcellulose medium containing GM-CSF, and interleukin-3. Colonies were evaluated and counted after 14 days, using an inverted microscope. The number of $CD34^+$ cells infused was measured both in purified BM and in leukapheresis products using a fluoresceinated MoAb (Beckton-Dickinson), as previously described. Data were gated on forward scatter lymphocytes and monocytes; ten thousand cells were evaluated for each sample. The number of T-lymphocytes after E-rosetting for T-cell depletion was measured using MoAb anti-CD3.[11,12]

In the remaining transplants, we performed the $CD34^+$ cell selection using the Clinimacs system (Miltenyi) because of the potentially better purity and recovery of cells that are virtually not affected by isolation procedures. The positive cells are specifically labeled with supermagnetic iron-dextran particles which are covalently conjugated to mouse anti-human CD34 antibody. After magnetic labeling, the Clinimacs system automatically passes cells through a separation column which is held in a strong permanent magnet. The magnetically labeled cells are retained within the column and separated from the unlabeled cells, which flow through. The retained cells are evaluated from the Clinimacs system by removing the column from the magnetic field, washing the cells and collecting them.

Results

Mobilization, collection and T-depletion of PBSCs

In the total apheresis collection, the mean number of $CD34^+$ cells was 13.42×10^6/kg. The mean number of PB $CD34^+$ cells collected after positive selection using the Isolex 300 System was 12.8×10^6/kg recipent's weight (range: 2-40) with a mean purity of 97.3%. After T-cell depletion using the E-rosetting method, the mean number of the infused $CD3^+$ cells was 0.385 $\times10^5$/kg (range, 0-0.98). Patient DCL received PBSC without any T-cell depletion, while patient CA received PBSC depleted with Campath-1M. When the Clinimacs system was used the mean number of PB $CD34^+$ cells collected was 14.05 $\times10^6$/kg recipent's weight (range: 8.1-20). No T-cell depletion was performed and the mean number of the infused $CD3^+$ cells was 0.53 $\times10^5$/kg (range, 0.06-1). The total apheresis mean number of CFU-GM infused was 8.1 $\times10^5$/kg of weight.

Collection and T-cell depletion of BM

When the Isolex 300 System was used, a mean of 11.4×10^6 bone marrow cells $CD34^+$/kg of recipient's weight was infused. All grafts were treated with Campath1M for T-depletion and the number of CD3+ cells was undetectable. When the Clinimacs system was applied for $CD34^+$ cell selection, the mean number of infused bone marrow $CD34^+$ cells was 9.85 $\times10^6$/kg of recipient's weight with a mean number of infused $CD3^+$ cells of 3.66×10^5/kg.

In the total bone marrow aspirates, the mean number of infused CFU-GM was 3.59×10^5/kg of recipient's weight.

Engraftment and chimerism

The 9 patients achieved peripheral blood neutrophil counts greater than 0.5×10^9/neutrophils/L at a mean of 14.6 days (range, 6 to 22 days). RFLP analysis of peripheral granulocytes and lymphocytes on day 14 confirmed engraftment of donor-CD3 derived cells in all cases. Granulocytes were autologous in 2 cases, split chimerism was observed in 1 case and complete chimerism in the remaining 6 cases.

Immune reconstitution

Phenotypic and functional analyses of post-transplant lymphocyte subsets were performed. In 8 patients who survived more than 120 days after BMT, the mean number of $CD2^+$ cells was 284/mm^3, that of $CD3^+$ cells 426/mm^3, $CD4^+$ 299/mm^3, $CD8^+$ 125/mm^3, $CD19^+$ 94/mm^3, and $CD16^+$ 132/mm^3.

Graft-vs.host disease

Six patients developed GVHD. One patient showed acute skin grade I GVHD, 2 patients developed grade II GVHD (1 skin and 1 skin plus gut) and 3 patients developed grade III acute skin GVHD; two of three patients with grade III GVHD were successfully treated with methylprednisolone. Only one patient developed acute grade IV skin GVHD after the first transplant and he was treated with steroids and ATG after the 2nd and 3rd transplants.

Toxicity and clinical outcome

The conditioning regimen was well tolerated by all patients. Eight of the nine patients are alive and well 1-37 months after BMT (mean follow up 7.5 months). One patient died of disseminated CMV infection early after BMT.

Discussion

Since the majority of children affected by PID do not have a compatible donor (40% have a matched unrelated donor[14] and only 10-15% have a healthy matched sibling, haploidentical BMT is the only curative treatment in this setting. Nevertheless, only children affected by SCID B^+ can be successfully treated (75% event-free survival) (EFS) by haploidentical BMT. Results of haploidentical BMT in other PID are still discouraging with an EFS of about 35-50%, due to failure to engraft or infectious complications related to late immunologic reconstitution.[5,15] Graft failure in patients affected by PID, conventionally conditioned with busulfan and cyclophosphamide in haploidentical BMT, could be related to insufficient myeloablation and immunosuppression.[16,17] Jabado *et al.*[18] have reported a new approach to enhance engraftment based on the use of a combination of two monoclonal antibodies: anti LFA-1 and anti-CD2. The results of their study suggest an increased rate of engraftment in non-SCID patients. Nevertheless, long-term results of this approach with regards to engraftment, infectious complications and immunologic post-transplant reconstitution remain to be evaluated. Among the several mechanisms claimed to explain the graft failure, insufficient conditioning regimen seemed to be the most likely as it lead s to insufficient inhibition of residual T-cell function.[19-21] An additional reason for failure of conventional haploidentical transplant could be the insufficient stem cell inoculum. In fact, it was shown in animal models that resistance to engraftment could be resolved if a large BM inoculum was used.[8] The rationale of using a conditioning regimen before BMT in PIDs is based on the need for immunosuppression and creation of "space" for the new hematopoesis.[22] However, these children receive a milder conditioning regimen than patients affected by hematologic malignancies. In patients affected by hematologic diseases, who cannot receive TBI and have diseases requiring an intensive conditioning regimen (acute lymphoblastic leukemia, myelodysplastic syndromes, thalassemia), the addition of thiotepa, a potent myeloablative drug, to the conventional busulfan-cyclophosphamide protocol, has been suggested.[23] In our group of patients, the conditioning regimen intensified by addition of thiothepa achieved good myeloablation. All patients tolerated the conditioning regimen well, without major toxicities. In haplodentical BMT profound T-cell depletion is required in order to avoid GVHD. However, an absence of T-lymphocytes in the graft prolongs immunodeficiency after BMT and may lead to an increased risk of opportunistic infections. In fact, in sharp contrast to HLA-matched BMT, a very delayed T-lymphocyte reconstitution, together with B-lymphocyte deficiency, has been observed in haploidentical BMT.[24] Transplantation of autologous peripheral blood stem cells is currently a well established method to reconstitute hematopoiesis in patients affected with lymphoma/leukemia or solid tumors, receiving a myeloablative preparatory regimen. Several reports showed how infusion of PBSC shortens the post-BMT aplastic phase with faster engraftment and allows prompt immunologic reconstitution as confirmed by conventional autologous BMT with marrow stem cells alone.[25,26] Recent data, regarding HLA-identical PBSC transplantation in hematologic malignancies, showed successful engraftment of all cell lineages without a significant increase in GVHD.[27] Studies performed in patients affected by leukemia show successful engraftment of a T-cell depleted haploidentical bone marrow by addition of recombinant human granulocyte colony-stimulating factor-mobilized peripheral blood progenitor cells to marrow inoculum, with accelerated hematopoetic recovery and without significant exacerbation of GVHD.[7,28] Nevertheless the series of patients treated in this report had advanced hematologic diseases, so data on long-term engraftment are not available. In PID, Friedrich *et al.*[29] reported 10 consecutive patients with SCID B^+ who received T-depleted haploidentical PBSC transplants with encouraging results. Again, longer follow-up of these patients is needed. Mobilization of peripheral $CD34^+$ cells by G-CSF and their collection by leukapheresis gives the possibility of obtaining at least $150\text{-}300\times10^6$ CD34 positive cells thus increasing the stem cell inoculum. Although peripheral blood $CD34^+$ cells show different surface markers from bone marrow cells,[30] studies in animals demonstrate that they are capable of inducing prompter immunologic reconstitution than bone marrow cells. Also, data on survival in long-term cultures of PBSC suggest their ability to repopulate bone marrow.[31,32] A study by Dunbar *et al.*[33] demonstrated long-term engraftment (18 months) in an autologous setting of both BM and PB stem cells using gene marking with two distinguishable vectors. Nevertheless no conclusive data are available to demonstrate that mobilized PBSC alone can guarantee long-term engraftment.[34-36] We have therefore combined the infusion of haploidentical T-cell-depleted bone marrow cells with added peripheral $CD34^+$ stem cells in order to increase the stem cell load. The choice not to positively select BM $CD34^+$ cells came from the consideration that, if the goal was to increase the stem cell inoculum, the yield after the posi-

tive selection is usually around 60% with an important decrease in the absolute number of $CD34^+$ cells. Engraftment was faster using our protocol than after conventional successful haploidentical BMT and we believe that this difference could be due to addition of PBSC. It is possible that the association of PBSC and BMSC could lead to a more effective competition between stem cells of host and donor origin increasing the engraftment rate. At the same time some authors claim that transplants with PBSC alone guarantee long-term engraftment, but the mean follow-up of patients does not exceed 10 years.[34] Therefore, at present we can only be sure that transplants including bone marrow cells guarantee a survival of several decades. The engraftment achieved in all our patients and the acceptable conditioning-related toxicity suggest that this approach could be successfully applied to children affected by PID who lack a matched donor.

References

1. Rosen FS. Report of a WHO Scientific Group: primary immunodeficiency disease, in Seligemann M (eds). Immunodeficiencies. Harwood Academic Publishers, 1994.
2. Buckley RH. Transplantation. In: Stiehm E.R, ed. Philadelphia:Saunders, 1996. Immunological aspects of pediatric illness, p.1014
3. Filipovich AH, Ramsay NKK, Porta F. Unrelated (URD) bone marrow transplantation for primary immunodeficiencies: an international review. Blood 1994; 84 (Suppl.1), 394 (Abs 1560).
4. Fischer A, Landais P, Friederich W, et al. Bone marrow transplantation in Europe for primary immunodeficiencies other than severe combined immunodeficiency: a report from the European Group for BMT and the European Group for Immunodeficiency. Blood 1994; 83:1149-55.
5. Friedrich W, Muller S, Schreiner T, Wiesneth M. Purified CD34+ blood stem cells from HLA-haploidentical parental donors for transplantation in SCID. The 6th Biennal Sandoz-Keystone Symposium on BMT "Blood cells and Bone Marrow Transplantation", Keystone, Colorado, USA Abs205, 1996.
6. Korbling M, Przepiorka D, Huh YO, et al. Allogenic blood stem cell transplantation for refractory leukemia and lymphoma: potential advantage of blood over marrow allografts. Blood 1995; 85:1659-65.
7. Aversa F, Tabilio A, Terenzi A, et al. Successful engraftment of T-cell depleted haploidentical "three-loci" incompatible transplants in leukemia patients by addition of recombinant human granulocyte colony-stimulating factor-mobilized peripheral blood progenitor cells to bone marrow inoculum. Blood 1994; 84: 3948-55.
8. Reisner Y, Martelli MF. Bone marrow transplantation across HLA barriers by increasing the number of transplanted cells. Immunology Today 1995; 16:437-40.
9. Aversa F, Terenzi A, Carrotti A, et al. Addition of Thiotepa improves results in T-cell depleted bone marrow transplant for advanced leukemia. Blood 1993; 82: 313a (Suppl.1).
10. Gasparini P, Martinelli G, Trabetti E, Ambrosetti A, Benedetti F, Pignatti PF. Bone marrow transplantation monitoring by DNA analysis. Bone Marrow Transplant 1990; 4:157-9.
11. Hale G, Waldmann H. Control of graft-versus-host disease and graft rejection by T-cell depletion of donor and recipient with Campath-1 antibodies. Results of matched sibling transplants for malignant diseases. Bone Marrow Transplant 1994; 13:597-611.
12. Reisner Y, Kapoor N, Kirkpatrick D, et al. Transplantation for severe combined immunodeficiency with HLA-A, B, D, DR incompatible parental marrow cells fractionated by soybean agglutinin and sheep blood cells. Blood 1993; 61:341-8.
13. Hardwick A, Kulcinski D, Mansour V, Ishizawa L, Law P, Gee A. Design of large-scale separation system for positive and negative immunomagnetic selection of cells using super-paramagnetic microspheres. J Hematother 1992; 1:379-81.
14. Beatty PG, Dahlberg S, Mickelson EM, et al. Probability of finding HLA-matched unrelated donors. Transplantation 1998; 45:714-8.
15. Fischer A, Landais P, Friedrich W, et al. European experience of bone marrow transplantation for severe combined immunodeficiency. Lancet 1990; 2:859-62.
16. Reisner Y, Lapidot T, Singer TS, Schwartz E. The host barrier in animal models of T-cell depleted allogenic bone marrow transplantation. In: Martelli MF, Grignani F, Reisner Y. Eds. T-cell depletion in allogenic bone marrow transplantation. Serono Symposia, review No.13. 1988. Rome, Italy, p 37.
17. Schwartz E, Lapidot T, Gozes D, Singer TS, Reisner Y. Abrogation of bone marrow allograft resistance in mice by increased total body irradiation correlates with eradication of host clonable T cells and alloreactive cytotoxic precursors. J Immunol 1987; 138:460-5.
18. Jabado N, Le Deist F, Cant A, et al. Bone marrow transplantation from genetically HLA-non identical donors in children with fatal inherited disorders excluding severe combined immunodeficiencies: use of two monoclonal antibodies to prevent graft rejection. Pediatrics 1996; 98:420-3.
19. Hansen JA, Anasetti C, Beatty PG, Martin PJ, Amos D, Thomas ED. Marrow transplants from HLA haploidentical donors: failure to engraft and late graft failure. In: Martelli MF, Grignani F, Reyner Y, Eds. T-cell depletion in allogenic bone marrow transplantation. Serono Symposia, review No.13, 1988. Rome, Italy, p. 37.
20. O'Reilly RJ, Collins NH, Kernan N, et al. Transplantation of marrow depleted of T cells by soybean lecitin agglutination and E-rosette depletion: major histocompatibility complex-related graft resistance in leukemic transplant recipients. Transplant P 1985; 17:455-8.
21. Kernan NA, Flomemberg N, Dupont B, O'Reilly R. Graft rejection in recipients of T-cell depleted HLA-non-identical marrow transplants for leukemia: Identification of host derived anti-donor allocytotoxic T-lymphocytes. Transplantation 1987; 43:842-6.
22. Blazar BR, Ramsay NKC. Pre-transplant conditioning with busulfan and cyclophosphamide for non-malignant disesaes. Transplantation 1985; 39:597-601.
23. Terenzi A, Lubin I, Lapidot T, et al. Enhancemenet of T-cell depleted bone marrow allografts in mice by Thiotepa. Transplantation 1990; 50:717-20.
24. Kook H, Goldman F, Padley D, et al. Reconstitution of the immune system after unrelated or partially matched T-cell depleted bone marrow transplantation in children: immunophenotypic analysis and factors affecting the speed of recovery. Blood 1996;

88;1089-97.
25. To LB, Haylock DN, Simmons PJ, Juttner CA. The biology and clinical use of blood stem cells. Blood 1997; 89:2233-58.
26. Roberts MM, To LB, Gillis D, et al. Immune reconstitution following peripheral blood stem cells transplantation, autologous bone marrow transplantation and allogenic bone marrow transplantation. Bone Marrow Transplantat 1993; 12:469-76.
27. Link H, Arseniev L, Bahre O, et al. Combined transplantation of allogenic bone marrow and CD34+ blood cells. Blood 1995; 86:2500-5.
28. Tabilio A, Aversa F, Regazzi T, et al. Hematopoietic and stromal reconstitution after haploidentical allogenic bone marrow transplantation. Blood 1996; 88 (Suppl 1):263a.
29. Friedrich W., Muller S., Schreiner T., Wiesneth M. (1996) Purified CD34+ blood stem cells from HLA-haploidentical parental donors for transplantation in SCID. The 6th Biennal Sandoz-Keystone Symposium on BMT "Blood cells and Bone Marrow Transplantation" Keystone, Colorado,USA Abs205.
30. To LB, Haylock DN, Dowse T, et al. A comparative study of the phenotype and proliferative capacities of peripheral blood (PB) CD34+ cells mobilized by four different protocols and those of steady-phase PB and marrow CD34+ cells. Blood 1994; 84:2930-9.
31. Yan XQ, Hartley C, McElroy P, Chang A, McCrea C, McNiece J. Peripheral blood progenitor cells mobilized by recombinant human granulocyte colony-stimulating factor plus recombinant rat stem cell factor contain long term engrafting cells capable of cellular proliferation for more than two years as shown by serial transplantation in mice. Blood 1995; 85:2303-7.
32. Drize N, Chertkov J, Sadovnikova E, Tiessen S, Zander A. Long-term maintenance of hematopoiesis by irradiated mice by retrovirally transduced peripheral blood stem cells. Blood 1997; 89:1811-7.
33. Dunbar CE, Cottler-Fox M, O'Shaughnessy JA, et al. Retrovirally marked CD34-enriched peripheral blood and bone marrow cells contribute to long term engraftment after autologous transplantation. Blood 1997; 85:3048-52.
34. Kessinger A. Autologous transplantation with peripheral blood stem cells: a review of clinical results. J Clin Apheresis 1990; 5:97-9.
35. Kessinger A, Smith DM, Strandjord SE, et al. Allogenic transplantation of blood-derived, T cell-depleted hemopoietic stem cells after myeloablative treatment in a patient with acute lymphoblastic leukemia. Bone Marrow Transplant 1989; 4:643-7.
36. Brito-Babapulle F, Bowcock SJ, Marcus RE, et al. Autografting for patients with chronic myeloid leukemia in chronic phase: peripheral blood stem cells may have a finite capacity for mantaining hematopoiesis. Br J Haematol 1989; 73:76-80.

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Treatment of childhood acute lymphoblastic leukemia after the first relapse: curative strategies

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ABSTRACT

Background and Objectives. **Treatment of recurrent childhood acute lymphoblastic leukemia (ALL) has been controversial in the last decade. Conventional intensive chemotherapy (CHEMO) can cure up to 30% of children who have relapsed after ALL: similar results have been obtained with autologous bone marrow transplantation (ABMT), but allogeneic bone marrow transplantation (AlloBMT) seems to be the best therapeutic option. In this review the authors point out the contribution of current strategy in the setting of children with ALL who experience a first relapse and should be offered optimal treatment in order to obtain the best disease-free survival (DFS). The principal objective of this issue is to reach a possible consensus on the more controversial points regarding factors considered strong predictors of the outcome of the relapsed patients, second-line chemotherapy, optimal timing and type of transplantation.**

Evidence and Information Source**. Data published in the literature over the last decade concerning early and late relapse in childhood ALL suggest that improvements in cure rates may be achieved by intensification of the relapse treatment both with chemotherapy and with different types of transplantation. An accurate search for Medline data has been performed in order to understand the risk-benefit ratio of aggressive therapy adopted in this setting.**

State of Art. **Modern first-line treatment protocols for childhood ALL have contributed to curing an ever larger number of patients but this strategy could be responsible for creating a more resistant leukemic clone at the time of systemic or extramedullary relapse. This hypothesis emerges from a number of single or multicenter experiences; thus clinical and biological features in relapsed patients are being studied crefully in order to understand which risk-directed second-line therapy should be best adopted. The BFM group classified ALL relapses as "very early", "early", or "late" according to the time from diagnosis to first relapse (i.e. <18, >18 and <30 or more than 30 months) and has shown that about 2/3 of the small fraction of children with late extramedullary relapses or late non T-marrow relapses or early combined non T-relapses can be rescued by chemotherapy; in contrast ALL early relapses or T-immunophenotype ALL relapses can be rescue only by AlloBMT. Since 1990 the AIEOP group adopted BFM-like first-line treatment and experienced similar situations for relapsed patients so that, even in absence of a real common relapse protocol, they went in the same direction as the BFM group as far as hematopoietic stem cell transplantation (HSCT) procedures and decision were concerned. A recent AIEOP study on the destiny of 192 consecutive patients with ALL in 2nd complete remission and not having an HLA suitable sibling donor is presented in this issue. The value of different HSCT procedures is addressed and the protection against a new relapse seems to be real, although, of course, the risk-benefit ratio should always be evaluated.**

Perspectives. **Very few prospective studies on the treatment of childhood ALL relapse have been set up in the last decade because of many difficulties regarding common second-line therapies, some reluctance versus HSCT in consideration of the transplant-related mortality and the so-called late effects. Additional modifications of allogeneic family and unrelated donor HSCT strategies and the promising results both of cord HSCT and auto-grafting methods including *in vitro* purging or post-transplant immunotherapy, are making transplantation procedures for ALL relapsed patients more appropriate and increasing confidence in their use. The possibility of performing common prospective international studies should be encouraged over the next years in order to elucidate an area of great research as is that of the treatment of childhood ALL relapse.**

Key words: ALL, treatment, childhood

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Despite the intensity of first-line chemotherapy in the treatment of childhood ALL, this strategy is not able to cure all the patients so that roughly 25% of ALL children suffer from relapse, mostly within the first 5 years from diagnosis.

Every year in Italy the AIEOP (*Associazione Ita-*

liana di Ematologia ed Oncologia Pediatrica) registers about 80 pediatric patients who experience a first ALL relapse but in the last decade it has not been possible to set up a common strategy for treating these relapses because some controversial points need to be solved.

One of the aims of this issue is to present the current opinion concerning the strategy for managing ALL relapse and to discuss the possibility of reaching a consensus on the following aspects: a) the optimal second-line treatment; b) the role of hematopoietic stem cell transplantation (HSCT); c) the possibility of designing a common relapse protocol and setting up a common data base in order to assess the results of a co-operative study homogeneously; d) the possibility of joining with other international prospective co-operative studies.

The optimal second-line treatment: rationale and general design

Results of treatment with chemotherapy in children after an ALL relapse remain unsatisfactory worldwide, especially in early relapses and in heavily pretreated patients. Only patients with a late relapse (>30 months after the diagnosis) or with an isolated extramedullary relapse have reasonable chances of cure after second-line chemotherapy. Results can be improved by allogeneic bone marrow transplantation (AlloBMT) when a suitable donor is available. Chemotherapy for first ALL relapse should thus take into account the treatment given as first-line therapy and BMT options.

Patients with ALL currently enrolled in AIEOP studies are treated with intensive chemotherapy schedules. About 80% of them are stratified as standard or intermediate risk patients; these patients receive protracted intensive chemotherapy and do not receive cranial radiotherapy unless there is central nervous system involvement at the diagnosis (<2%). High risk patients (20%) also receive intensive rotational chemotherapy and cranial radiotherapy.

Protocols used in the last decade have been very similar to BFM protocols[1,2] and thus the BFM experience in the treatment of ALL relapses provides important information for AIEOP too. Since 1983 the BFM group has been treating ALL relapses with intensive chemotherapy blocks of non-cross-resistant antineoplastic agents, cranial or craniospinal radiotherapy and maintainance therapy.[3,4] The BFM group has classified ALL relapses as *very early*, *early* or *late* according to time from diagnosis to relapse (< 18; > 18 and < 30; > 30 months, respectively) and has shown that about 2/3 of the small fraction of children with late extramedullary relapse and about 1/3 of those with early extramedullary relapses or late non-T marrow relapses or early combined non-T relapses can be rescued by chemotherapy; conversely, bone marrow relapses occurring earlier or with T-immunophenotype can be rescued only by BMT. The concept that after intensive front-line chemotherapy only late ALL relapses have good chances of being rescued by chemotherapy is confirmed by results obtained by other institutional or co-operative groups.[5,6]

The AIEOP approach (1998 AIEOP guidelines) for treatment of ALL relapses has been developed in this context. According to this strategy ALL relapses are defined as standard risk (non-T-ALL relapsing >30 months after diagnosis), intermediate risk (extramedullary relapses occurring <30 months after diagnosis) or high risk (bone marrow relapses occurring < 30 months after the diagnosis and all relapses of T-ALL).

The large majority of standard risk ALL relapses occur in patients treated with front-line therapies for non-high risk ALL. These patients are not heavily pre-treated and have a high probability (about 90%) of obtaining a second CR after a treatment with standard ALL front-line chemotherapy. Accordingly, induction therapy for standard risk ALL relapse consists of four weeks of prednisone, four weekly doses of vincristine and idarubicin, 8 doses of asparaginase (every three days) and intrathecal chemotherapy; after achieving complete remission, three intensive chemotherapy blocks are administered as consolidation therapy; in patients not undergoing BMT, treatment is continued with a reinduction phase (i.e. BFM protocol II modified in that idarubicin is substituted for daunomycin), cranial radiotherapy and maintenance with rotational combination chemotherapy[5-7] for a total treatment duration of two years.

In the AIEOP experience, patients with intermediate risk ALL relapses (early or very early extramedullary relapses) have a poor outcome and are thus treated with the same chemotherapy approach adopted for high risk ALL relapses. Induction therapy for these patiens consists of a single high dose of idarubicin (40 mg/m^2) and high dose arabinoside cytosine (ARA-C) (3 gr/m^2/day x 5).[6] Seventy-four patients have been treated with this schedule with 82% of them obtaining a second CR. Early mortality and resistance rates have been respectively 10% and 8%. Although CR rate is satisfactory, these patients have a very high probability of developing a second very early relapse. While waiting for BMT, a consolidation phase consisting of six weeks of chemotherapy (prednisone, vincristine, L-asparaginase (L-ASP), teniposide, and intrathecal therapy) used in the R11 study as induction therapy,[5] followed by three intensive

chemotherapy blocks has been administered to these patients. As expected, however, 25% of the patients who achieved a second CR relapsed in this phase, suggesting that treatment used may be inadequate and that BMT should be performed as soon as possible. For patients not undergoing BMT and remaining in CR, chemotherapy after consolidation phase is continued as for standard risk patients.

Role of different types of HSCT: past, present and future

The decision to perform allogeneic matched family or unrelated donor (MFD /MUD) HSCT, autologous peripheral or marrow or cord blood HSCT depends on many factors which can be considered strong predictors of the outcome of the relapsed patients as has emerged from a number of literature reports that deserve some comments.

Sites and time of relapse

Different sites of relapse and the tempo of the relapse may be the most important factors predicting the outcome after a first relapse.

Except for *late isolated extramedullary relapse* (over 6 months from therapy withdrawal) in which chemotherapy alone plays a favorable role,[8-10] all other kinds of relapses (isolated/combined medullary particularly) taking place during therapy or within 30 months of the diagnosis of ALL seem to benefit more from different HSCTs than chemotherapy.

The overall probability of disease-free survival (DFS), as derived from multicenter studies over the last 10 years, ranges from 30 to 60%[11-21] with some advantage for AlloMFD HSCT compared with other kinds of HSCT[10-20]). The AIEOP and GITMO (*Associazione Italiana di Ematologia ed Oncologia Pediatrica e Gruppo Italiano Trapianto di Midollo Osseo*) groups demonstrated a significant advantage of AlloMFD HSCT over chemotherapy only in early medullary relapse patients[18] and again an advantage in terms of DFS between AlloMFD HSCT and autologous HSCT for childhood ALL in 2nd complete remission treated with the same conditioning regimen.[20]

The difference between chemotherapy and AlloMFD HSCT for patients who experienced a late marrow relapse (45% DFS vs 65%),[15-17] i.e. over 30 months from diagnosis, did not was evident but not statistically significant.

In summary the current opinion is that the earlier the relapse the more difficult it is to obtain and maintain a second CR. In this sense transplantation procedures should be considered as elective therapeutic options in order to eradicate resistant disease.

Immunophenotype, cytogenetics, biological characteristics of relapsed patients

There is no doubt that patients with T-cell ALL relapses have a poor prognosis. While any kind of chemotherapy is unsuccessful for them, some promising results can be obtained with transplant strategies following this priority *cascade*: AlloMFD HSCT, MUD or Haplo HSCT, cord blood HSCT, autologous HSCT.

One biological predictor of negative outcome could be the number of peripheral blood blast cells (≥1/µL to <10,000 µL or >10,000 µL) at the time of relapse according to POG or BFM group experience[4,21] so that one could justify AlloMFD or MUD HSCT even in patients with *late relapse*. On the other hand the absence of peripheral blast cells at relapse has been associated with a 10-year EFS of 64% suggesting that these patients are not candidates for HSCT.[22]

The MLL rearrangement or a BCR-ABL positive relapse makes patients elective candidates for the above *cascade* of HSCT due to the documented particularly poor prognosis if patients are treated by chemotherapy only.[23] Tel-AML1 fusion, the molecular characteristic of approximately 25% of B-lineage ALL patients at diagnosis,[24] should be otherwise considered as a good outcome marker even in relapsed patients. This particular subset of patients experience very late relapse, get and maintain an excellent 2nd CR undergoing chemotherapy only.

Availability and role of MUD HSCT compared with other HSCTs

AlloMFD HSCT is currently a limited transplant option because only 20 to 30% of relapsed patients have an HLA A-B and DR identical sibling donor. HSCT from a MUD has become a feasible procedure capable of curing a significant proportion of children with ALL lacking an HLA identical family donor.

Recently Balduzzi,[25] Davis,[26] Oakhill,[27] Heslop[28] and others reported a 2-3 year EFS between 40% to 53% for ALL children in 2nd CR treated with MUD HSCT.

In particular in Oakhill's study there was no significant difference in outcome between patients who received fully matched unrelated marrow and those who received partially mismatched marrow. The study by Heslop *et al.* demonstrated that there was no difference between ALL relapsed children undergoing AlloMFD HSCT and MUD HSCT.

All these studies reported the outcome of selected cohorts of patients with ALL who actually received a MUD HSCT. Recently the AIEOP reported on the outcome of 192 consecutive children with 2nd CR ALL for whom the search

for a MUD was activated. One of the aims of this study was to overcome the biases related to the allocation of patients to different therapeutic options.[29] The probability of finding a MUD within 6 months after search activation was 21% (SE 5) and 37% (SE 5) before and after 1995, respectively (p = 0.01). The major obstacle to the success of the search was a second relapse. The 6-month probability of relapse during the search was 39% (SE 3.9). Treatment effectively assigned to patients was dependent not only on donor availability, but also on the course of the disease during the search: 83 out of 192 children found a MUD but only 73 were given a MUD HSCT; the remaining 10 children lost their eligibility to this procedure because of progressive disease and died. Nineteen out of the 73 (38%) children undergoing a MUD HSCT survived in complete remission. Of the 109 patients who did not find a suitable donor, 70 underwent chemotherapy alone but only 5 of them (7%) survived without leukemia, while the remaining 39 children were given other types of HSCTs and only 13 of them survived in complete remission.

Recently, cord blood HSCT has been shown to be feasible and has yielded some encouraging preliminary results[30,31] as is described in more detail in this issue by Locatelli.

In this setting the evidence of quite similar results between cord blood HSCT and MUD HSCT, when applied to patients with malignancies, is particularly interesting.

For selected relapsed patients for whom AlloMFD HSCT or MUD HSCT is not possible, autologous HSCT may offer a chance of cure. Retrospective single center studies[32] demonstrated the efficacy of autologous HSCT for late relapsed B-precursor ALL (over 2 years from diagnosis) with a 3-year EFS of 53% and the same result was confirmed afterwards by the same group[33] when autologous HSCT was compared with AlloMFD HSCT (53% EFS vs. 47%).

Autologous HSCT for ALL children in 2nd CR performed by the AIEOP group[34] yielded an 8-year EFS of 34% indicating, also by univariate analysis, an advantage for isolated extramedullary relapse vs BM relapse (68% EFS vs 18%) and for patients undergoing TBI conditioning regimens vs. no TBI (48% EFS vs15%).

An Italian single center study[35] recently demonstrated a promising result in ALL children in 2nd CR when rescued both with autologous HSCT and a particularly efficient purging technique such as monoclonal antibodies and double selection of CD34 peripheral blood stem cells: the PCR negative infused product gave a 2-year probability of EFS of 89%. Our experience addresses the possible advantage of autologous HSCT procedures when additional modifications of autografting methods, including *in vitro* purging or post-transplant immunomodulation, are applied.

A BFM matched–pair analysis in childhood ALL in 2nd CR treated by autologous HSCT and chemotherapy demonstrated an overall 9-year EFS of 32% vs. 26%[36] Multicenter retrospective studies comparing MUD HSCT vs. autologous HSCT[37,38] in a mixted 2nd CR ALL series (adults and children) came to controversial conclusions due to the excessive toxicity in unrelated transplantation which limited the apparent superiority of this former procedure vs autologous HSCT.

Recently it has been reported that matching HLA class I and class II alleles of the donor and recipient can significantly improve the outcome after MUD HSCT,[39] but the best transplantation strategy is to carry out this transplant possibly within 3-4 months from the beginning of the search. This is the reason why, in the absence of a fully compatible donor, a one antigen mismatched donor is acceptable too.[40] If no MUD or cord blood units are available within 3 to 5 months from the beginning of the search , a haploidentical HSCT should be offered to patients who are in second remission after an early relapse.

Pre- and post-HSCT factors which can play some role in the outcome of childhood ALL in 2nd CR

The quality of 2nd CR obtained after an intensive second line therapy remains the golden standard for applying successfully whatever subsequent consolidation therapy (chemotherapy or any kind of HSCT). Molecular monitoring of minimal residual disease is now available and could have an important role in future strategies for treating relapses.[41]

Transplant procedures such as conditioning regimens including total body irradiation together with high doses of several drugs (cytoxan, VP-16, Ara-C, vincristine) should be tested in multicenter prospective and/or randomized studies in order to understand the role of different drugs in eradicating the leukemic clone.

Careful evaluation of the best conditioning regimen to adopt should be done by transplant teams in order to lessen so-called transplant-related mortality and *late effects*. In this respect, continuous improvement of support treatment over the last 10 years seems to have provided better results in terms of short- and long-term quality of life, and indeed non-myeloablative regimens represent a recent field of interest in order to decrease transplant toxicity and mortality in particular subsets of patients.

Recently some clinical studies have shown that allogeneic engraftment can be accomplished by non–myeloablative regimens based predominantly on fludarabine and /or low–dose TBI.[42] These experiences are giving rise to the concept that allogeneic non-myeloablative transplants are much better tolerated than standard conditioning regimens, both in adults and in children. Limited toxicity, prompt engraftment, and stable and full chimerism were obtained in more than 90% of the recipients. To date this approach has been adopted in advanced stages of malignant diseases and in heavily pretreated patients; for these reasons its real capacity to control the underlying disease has been probably underestimated. The few data concerning patients with early relapse and minimal residual disease are more interesting and similar to the data obtained with myeloablative regimens.[43,44] One can hypothesize that children with late medullary relapse or isolated extramedullary relapse and an HLA identical family donor might undergo a non-myeloablative regimen.

Last but not least an emerging favorable factor in transplantation strategy could be the increase of graft-versus-leukemia effect, which has also been demonstrated by the AIEOP group,[45] by the immunomodulatory effect of the graft-versus-host in the early post-transplant phase. The possibility of eradicating minimal residual disease was in fact demonstrated in patients receiving low dose cyclosporin A (CyA) vs. standard dose CyA as GVHD prophylaxis which resulted in a decrease of the overall relapse rate.

Possibility of setting up a common relapse protocol and data base for the AIEOP group

During the last 10 years the first line treatment for children with newly diagnosed ALL has been administered according to three consecutive AIEOP protocols, which adopted BFM-based chemotherapy.[46-48] Between January 1988 and April 1998, 3,015 consecutive children were centrally registered at diagnosis and followed up yearly. By December 1999, 857 consecutive children had experienced an event, including in 581 cases an isolated bone marrow relapse. In most cases salvage treatment was given on the basis of single institution decisions according to either AIEOP or BFM guidelines for treatment of relapsed patients.[3, 49-51] If, in the future, we be can set up a common relapse protocol it will be easier for the AIEOP Registry to collect data prospectively on relapsed patients[52,53] and to improve the results.

The principal eligibility criteria for entering such a study should be the attainment of second CR after a common relapse protocol. Subsequently, a treatment arm such as chemotherapy or any kind of HSCT should be assigned according to common decisions based mainly on time from diagnosis to relapse and on site of relapse. Such a collaborative study requires a good relationship between clinicians involved in the front-line protocols and in the transplantation program.

Possibility of joining with other International study groups for a common co-operative relapse study

In 1996 the *Pediatric Working Party of the European Group for Bone Marrow Transplantation* (EBMT) reached a consensus on HSCT indications for childhood ALL.[54] The need for a prospective relapse study in childhood ALL is urgent and this is the reason why the EBMT and the I-BFM-SG tried to set up this kind of project last year. The common eligibility criteria and the common conditioning regimen to apply for AlloBMT patients constitute the principal conditions for entering patients in this study. The priority *cascade* of different kinds of transplantations (AlloMFD, MUD, haplo identical, cord blood and autologous HSCT) must be accepted by all the participating centers in order to validate all the results coming from different procedures according to an *intention-to-treat* analysis. A common data base is going to be set up and should pool some relevant information on all the patients eligible for the relapse protocol so that each group will periodically provide the basic data to the co-ordination unit in order to make the data management feasible. The principal aim of a co-operative prospective study like that should be to avoid having many different experiences which can result in a waste of time and effort. We think that the actual existence of co-operative study groups such as the EBMT, I-BFM-SG and AIEOP will allow this common relapse study to be carried out successfully as were the studies on myelodisplastic syndromes and childhood very high risk ALL in first CR.

References

1. Conter V, Aricò M, Valsecchi MG, et al. Intensive BFM chemotherapy or childhood ALL: interim analysis of the AIEOP-ALL 91 Study. Haematologica 1998; 83: 791-9.
2. Reiter A, Schrappe M, Ludwig WD, et al. Chemotherapy in 998 unselected childhood acute lymphoblastic leukaemia patients. Results and conclusions of the multicenter trial ALL BFM 86. Blood 1994; 84:3122-33.
3. Henze G, Fengler R, Hartmann R, et al. Six – year experience with a comprhensive approach to the treatment of recurrent childhood acute lymphoblastic leukaemia (ALL-REZ BFM 85). A relapse study of the BFM Group. Blood 1991; 78:1166-75.
4. Buher C, Hartmann R, Fengler R, et al. Peripheral blast count at diagnosis of late isolated bone marrow

relapse or childhood acute lymphoblastic leukaemia predict response to salvage chemotherapy and outcome. BFM relapse study group. J Clin Oncol 1996; 14:2812-7.
5. Testi AM, Moleti ML, Giona F, et al. A single high dose of Idarubicin combined with high-dose-ARA-C (MSKCC ALL-3protocol) in adult and paediatric patients with acute lymphoblastic leukaemia. Experience of the University "La Sapienza" of Rome. Haematologica 1997; 82 (Suppl. 5):19-22.
6. Rivera GK, Hydson MM, Liu Q, et al. Effectiveness of intensifies rotational combination chemotherapy for late hematologic relapse of childhood acute lymphoblastic leukaemia. Blood 1996; 88:831-7.
7. Weiss M, Maslak P, Megerian L, Schenberg DA . Phase I trial of a single high dose of IDARUBICIN combined with high dose CYTARABINE (ARA-C) as induction therapy in relapsed or refractory acute lymphoblastic leukaemia (ALL). Proceedings ASH, 1995; Supp. 1, 786a.
8. Uderzo C, Zurlo MG, Adamoli L, et al. Treatment of isolated testicular relapse in childhood acute lymphoblastic leukaemia: an Italian multicenter study. J Clin Oncol 1990; 8:672-7.
9. Borgmann A, Hartmann R, Schmid, et al. Isolated extramedullary relapse in children with ALL: a comparison between treatment results of chemotherapy and bone marrow transplantation. BFM relapse Study Group. Bone Marrow Transplant 1995; 15:515-21.
10. Ritchey AK, Pollock BH, Lauer SJ, et al. Improved survival of children with CNS relapse of acute lymphoblastic leukaemia: a Paediatric Oncology Group study. J Clin Oncol 1999; 12:3745-52.
11. Barrett AJ, Horowitz MM, Pollock BH, et al. Bone marrow transplants from HLA identical siblings as compared with chemotherapy for children with acute lymphoblastic leukaemia in a second remission. N Engl J Med 1994; 331:1253-8.
12. Coccia PF, Strandjord SC, Warkentein PL, et al. High dose cytosine arabinoside and fractionated total body irradiation. An improved regimen for bone marrow transplantation of children with acute lymphoblastic leukaemia in remission. Blood 1988; 71:888-93.
13. Bordigoni P, Esperou H, Souillet G, et al. Total body irradiation –high dose cytosine arabinoside and melphalan followed by allogeneic bone marrow transplantation from HLA-identical siblings in the treatment of children with acute lymphoblastic leukaemia after relapse while receiving chemotherapy: a Société Francaise de Greffe de Moelle study. Br J Hematol 1998; 102:656-65.
14. Brochstein JA, Kernan NA, Groshen S, et al. Allogeneic bone marrow transplantation after Hyperfractionated total body irradiation and cyclophosphamide in children with acute leukaemia. N Engl J Med 1987; 317:1618-24.
15. Chessels J, Leiper A, Plowman P, et al. Bone marrow transplantation has limited role in prolonging second marrow remission in childhood acute lymphoblastic leukaemia. Lancet1986; 1:1239-41.
16. Hoogerbrugge PM, Gerritsen EJA, Does-van den Berg A, et al. Case–control analysis of allogeneic bone marrow transplantation versus maintenance chemotherapy for relapsed ALL in children. Bone marrow transplant 1995; 15:255-9.
17. Dopfer R, Henze G, Bender-Gotze C, et al. Allogeneic bone marrow transplantation for childhood acute lymphoblastic leukaemia in second remission after intensive primary and relapse therapy according to the BFM and Co ALL protocols: results of the German Cooperative study. Blood 1991; 78:2780-4.
18. Uderzo C, Valsecchi MG, Bacigalupo A, et al. Treatment of childhood acute lymphoblastic leukaemia in second remission with allogeneic bone marrow transplantation and chemotherapy: ten –year experience of the Italian bone marrow transplantation group and the Italian Pediatric Hematology Oncology association. J Clin Oncol 1995;13:352-8.
19. Zecca M, Pession A, Messina C, et al. Total body irradiation, thiotepa, and cyclophosphamide as a conditioning regimenfor children with acute lymphoblastic leukaemia in first or second remission undergoing bone marrow transplantation with HLA-identical siblings. J Clin Oncol 1999; 17:1838-46.
20. Uderzo C, Rondelli R, Dini G, et al. High-dose vincristine, fractionated total-body irradiation and cyclophosphamide as conditioning regimen in allogeneic and autologous bone marrow transplantation for childhood acute lymphoblastic leukaemia in second remission: a 7-year Italian multicentre study. Br J Haematol 1995; 89:790-7.
21. Sander JE, Thomas ED, Buckner CD, et al. Marrow transplantation for children with acute lymphoblastic leukaemia in second remission.Blood 1987; 79:517-26.
22. Rivera GK, Sandowitz PD, Smith SD, et al. Treatment of late bone marrow relapse in children with acute lymphoblastic leukaemia: a POG study. Blood 1993; 81:602-9.
23. Biondi A, Cimino G, Pieters R, Pui CH. Biological and therapeutic aspects of infant leukaemia. Blood 2000; 96:24-33.
24. Mclean TW, Ringold S, Neuberg D, et al. Tel/AML1 dimerizes and is associated with a favorable outcome in childhood acute lymphoblastic leukaemia. Blood 1996; 88:4252-8.
25. Balduzzi A, Gooley T, Anasetti C, et al. Unrelated donor marrow transplantation in children. Blood 1995; 86:3247-55.
26. Davies SM, Wagner JE, Shu Xo, et al. Unrelated donor marrow transplantation for children with acute leukaemia. J Clin Oncol 1997; 15:557-65.
27. Oakhill A, Pamphilon DH, Potter MN, et al. Unrelated donor marrow transplantation for children with relapsed acute lymphoblastic leukaemia in second complete remission. Br J Haematol 1996; 94:574-8.
28. Hongeng S, Krance RA, Bowman LC, et al. Outcomes of transplantation with matched sibling and unrelated donor bone marrow in children with leukaemia. Lancet 1997;350:767-71.
29. Dini G, Valsecchi MG, Micalizzi C, et al. on behalf of AIEOP and GITMO: Esito dei pazienti con leucemia linfoblastica acuta per cui è stata avviata la ricerca di un donatore di midollo osseo volontario. Riv Ital Pediatr 1999 ; 25:15-7.
30. Rubinstein P, Carrier C, Scaradovan A, et al. Outcomes among 502 recipients of placental blood transplants from unrelated donor. N Engl J Med 1998; 339:1565-77.
31. Locatelli F, Rocha V, Chastang C, et al. Factors associated with outcome after cord blood transplantation in children with acute leukaemia. Blood 1999; 93:3662-71.
32. Billet AL, Kornmehl E, Tarbell NJ, et al. Autologous bone marrow transplantation after a long remission for children with recurrent ALL. Blood 1993; 6:1651-7.
33. Parson SK, Castellino SM, Lehmann LE, et al. Relapsed acute lymphoblastic leukemia: similar outcomes for autologous and allogeneic marrow transplantation in selected children. Bone Marrrow Transplant 1996; 17:763-8.
34. Messina C, Cesaro S, Rondelli R, et al. Autologous bone marrow transplantation for childhood acute

lymphoblastic leukaemia in Italy. Bone Marrow transplant 1998; 21:1015-21.
35. Balduzzi A, Gaipa G, Bonanomi S, et al. Purified autologous grafting in childhood acute lymphoblastic leukaemia in second remission: evidence for long-term clinical and molecular remission. Leukaemia 2000, in Press.
36. Borgmann A, Schmid H, Hartmann R, et al. Autologous bone marrow transplants compared with chemotherapy for children with ALL in a second complete remission: a matched pair analysis. Lancet 1995; 346:873-6.
37. Busca A, Anasetti C, Anderson G, et al. Unrelated donor or autologous marrow transplantation for treatment of acute leukaemia. Blood 1994; 83:3077-86.
38. Weisdorf D, Billet A, Hannan P, et al. Autologous versus unrelated donor allogeneic marrow transplantation for acute lymphoblastic leukaemia. Blood 1997; 8:2962-8.
39. Petersdorf EW, Gooley TA, Anasetti C, et al. Optimizing outcome after unrelated marrow transplantation by comphrehensive matching of HLA class I and II alleles in the donor and recipient. Blood 1998; 92:3515-20.
40. Souliet G, Rey S, Bertrand Y, et al. Outcome of unrelated bone marrow donor searches in 174 children resulting in 45 patients transplanted in the HLA-matched and –mismatched situation. Bone Marrow Transplant 2000; 26:31-43.
41. Kersey JH. Fifty years of studies of the biology and therapy of childhood leukaemia. Blood 1997; 90:4234-51.
42. Slavin S, Nagler A, Naparstek E, et al. Non-myeloablative stemcell transplantation and cell therapy as an alternative to conventional bone marrow transplantation with lethal cytoreduction for the tretment of malignant and non malignant hematologic diseases. Blood 1998; 91:756-63.
43. Martino R, Sierra J. Allogeneic hematopoietic stem cell transplantation after immunosuppressive but non-myeloablative conditioning: "miniallografts" are no small matter. Haematologica 1998; 83:865-7.
44. Carella AM, Champlin R , Slavin S, et al. Mini-allografts: ongoing trials in humans. Bone Marrow transplant 2000; 25:345-50.
45. Locatelli F, Zecca M, Rondelli R, et al. Graft versus host disease prophylaxis with low-dose cyclosporine A reduces the risk of relapse in children with acute leukaemia given HLA-identical sibling bone marrow transplantation: results of a randomized trial. Blood 2000; 95:1572-9.
46. Conter V, Aricò M, Valsecchi MG, et al. Extended intrathecal methotrexate may replace cranial irradiation for prevention of CNS relapse in intermediate risk ALL children treated with BFM-based intensive chemotherapy. J Clin Oncol 1995 ;13:2497-502.
47. Vecchi V, Aricò M, Basso G, et al. Risk-directed therapy for childhood acute lymphoblastic leukaemia. Cancer 1993 ;72:2517-24.
48. Conter V, Aricò M, Valsecchi MG, et al. Intensive BFM chemotherapy for childhood ALL: interim analysis of the AIEOP-ALL 91 study. Haematologica 1998; 83: 791-9.
49. Buhrer C, Hartmann R, Fengler R, et al. Superior prognosis in combined marrow relapse compared to isolated bone marrow relapse salvage therapy of childhood acute lymphoblastic leukaemia. Med Ped Oncol 1993; 21:470-6.
50. Rossi MR, Masera G, Zurlo MG, et al. Randomized multicentric italian study on two treatment regimens for marrow relapse in childhood acute lymphoblastic leukaemia. Pediatr Hematol Oncol 1986;3:1-9.
51. Giona F, Testi AM, Annino L, et al. Treatment of primary and relapsed acute lymphoblastic leukaemia in children and adults: the GIMEMA/AIEOP experience. Gruppo Italiano Malattie Ematologiche Maligne dell'Adulto. Associazione Italiana Ematologia ed Ocologia Pediatrica. Br J Haematol 1994; 86:55-61.
52. Pession A, Rago S, De Rosa M, et al. Modello di gestione dei dati: studio nazionale AIEOP per la leucemia acuta linfoblastica del bambino. Haematologica 1987; 72:249-54.
53. Pession A, Rondelli R, Paolucci P, et al. Hematopoietic stem cell transplantation in childhood: report from the Bone Marrow Transplantation (BMT) group of the Associazione Italiana Ematologia Oncologia Pediatrica (AIEOP). Haematologica 2000; 85:638-43.
54. Dini G, Cornish JM, Gadner H, et al. Bone marrow transplant indications for childhood leukemias: achieving a consensus. The EBMT Pediatric Diseases Working Party. Bone Marrow Transplant 1996; 18:4-7.

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Surveillance of cytomegalovirus infections in bone marrow transplant in Trieste: seven years' experience

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ABSTRACT

Forty-five consecutive patients submitted to a bone marrow transplant (BMT) were followed up weekly in order to evaluate the incidence of cytomegalovirus (CMV) infections on the basis of CMV antigenemia and polymerase chain reaction. All but one transplanted patients engrafted; fourteen patients out of these were CMV antigenemia positive after 16-184 days (median 32.5, mean 43.4) with an 31.8% incidence. CMV infections were associated with graft-versus-host disease and immunogenetic relationship between the donor and the recipient. No CMV infection was detectable in autologous transplants while antigenemia was demonstrated in 3/11 and 6/7 patients with BMT from respectively mismatched related and matched unrelated donors.

Key words: Cytomegalovirus, bone marrow transplant, surveillance, PCR.

Viral infections are important complications after allogeneic bone marrow transplantation (BMT) and have a heavy impact on morbidity and mortality. The most frequent and clinically relevant are related to members of the Herpesvirus family.[1,2] In fact, a cytomegalovirus (CMV) infection is demonstrable in 50-70% of allogeneic BMT recipients and one third of these develops interstitial pneumonia (IP) with a case fatality ratio of 85%.[3-5] The main risk factors for viral complications in allogeneic BMT are latent viral infections of donors and of recipients, donor HLA compatibility, conditioning and graft-versus-host disease (GVHD).[6, 7] Post-transplant immune reconstitution after BMT is characterized by an early leukopenic granulocytopenic phase lasting one month, followed by a period between days 30 – 100 with a marked lack of T-cell function and characterized by acute GVHD; significative immune competence is recovered by the sixth month.[8-10] Curiously viral infections are not evenly distributed in the post-transplant period: the most frequently occurring infections in the first month are HSV oral mucositis and respiratory virus infections; CMV infections are the main complications in the period ranging from day 30 to 100 although they have recently begun to frequently demonstrated later with similar mortality.[11, 12] Epstein-Barr virus reactivations are relatively frequent between the 2nd and 4th months while *Varicella Zoster* virus reactivations between the 4th and 12th month.

In recent years preventive measures and preemptive therapy have reduced CMV interstitial pneumonia associated mortality from 80% to 10-20%;[13,14] this underlines the relevance of virologic monitoring in order to detect systemic CMV infections early. An emerging problem is the selection of mutant strains resistant to the antivirals used in prophylaxis.[15]

In this study we report the incidence of CMV infections in a two-year follow-up of forty-five consecutive BMT patients evaluated by pp65 antigenemia and polymerase chain reaction (PCR) investigations.

Design and Methods

Patients

Forty-five consecutive patients who underwent BMT performed in the BMT Unit of our hospital between 10 January 1992 and 25 November 1999 were included in the study. Clinical data of the 44 engrafted BMT patients are reported in Table 1. In brief, 21 were female and 23 male, their median age was 10.6 years (11.6 F, 8.7 M); indications for BMT included acute lymphoblastic leukemia, acute myelogenous leukemia, chronic myeloid leukemia, solid tumours, autoimmune diseases, myelodysplastic syndromes, and thalassemi. Fourteen patients needed a further BMT for relapse or lack of engraftment.

All patients were screened for CMV antigenemia weekly until day +100; samples of polymorphonuclear cells were store at –20°C for further analysis by PCR amplification.

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Table 1. Summary of clinical data of the 44 engrafted patients.

		N°	CMV infections	p#
Sex				
	Male	23	9	0.223
	Female	21	5	
Age				
(years)	1-4	11	3	
	5-9	10	2	
	10-14	12	4	0.595
	15-19	7	4	
	>19	4	1	
CMV serol. Pre-transpl.				
	Negative	12	2	0.186
	Positive	32	12	
CMV serol. Donor				
	Negative	10	2	
	Positive	32	11	0.763
	Unknown	2	1	
Diagnosis				
	AL	1	0	
	ALL	15	8	
	AML	4	2	
	CML	4	2	0.121
	ST	7	0	
	AID	6	0	
	GEN	6	2	
	MDS	1	0	
Donor				
	Autologous	14	0	0.001
	MRD	11	3	
	Haploidentical	12	5	
	MUD	7	6	
GVHD				
	No	25	2	0.001
	Acute I	8	5	
	Acute II-IV	9	7	
	chronic	2	0	

AL: acute leukemia; ALL: acute lymphoblastic leukemia; AML: acute myelogenous leukemia; CML: chronic myeloid leukemia; ST: solid tumor; AID: autoimmune disease; GEN: genetic disease; MDS: myelodysplastic syndrome. MRD: matched related donor; MUD: matched unrelated donor; #Pearson Chi-square test.

CMV antigenemia

The antigenemia assay was performed using a CINApool kit (Argene-Biosoft) according to the manufacturer's instructions. Specimens were processed within 4 h of collection and a positive result was defined as ≥ 2 cells with fluorescent nuclei in duplicate spots containing 2×10^5 white blood cells.

PCR

A subset of 60 samples of polymorphonuclear DNA was examined by PCR. Amplification of a 435 bp fragment in the CMV major immediate early gene and of a 400 bp region in the late antigen gene was performed by single step PCR using primers MIE4–MIE5 and LA1–LA6[16] respectively in a Perkin Elmer thermal cycler.

After a 5' DNA denaturation step, 45 cycles of denaturation at 95°C for 30", primer annealing at 60°C for 30" and DNA elongation at 72°C for 30" were followed by a final DNA extension at 72°C for 5'. Each run included one positive viral control (AD169) and two negative controls.

Table 2. Comparison of CMV detection in PMN by pp65 antigen assay and PCR.

		PCR	
	positive	negative	total
Antigen assay			
positive	19	0	19
negative	1	40	41
total	20	39	60

The amplified products were demonstrated by gel electrophoresis, stained with ethidium bromide and examined on an ultraviolet transilluminator.

Statistical analyses

Differences in proportions between groups were examined using Pearson's chi-squared test and Fisher's exact test. Statistical calculations was performed using SPSS 10 software.

Results

The comparison between the results obtained with antigenemia and PCR techniques in a subset of 60 samples is reported in Table 2. PCR detected all 20 infected patients while antigenemia resulted negative in a PCR positive sample, thus giving a test sensitivity of 95%. The PCR positive-antigenemia negative result occurred in a patient with low levels of CMV in white blood cells and he become pp65 positive one week later.

Of the 44 engrafted patients 14 were CMV positive by the antigenemia test. None of the 14 autologous BMT patients developed a CMV infection while the incidence was 46.7% in the group of patients receiving an allogeneic BMT. In detail 3 out 11 recipients of a transplant from an HLA matched related donor, 5 out 12 from a haploidentical donor and 6 out 7 from a matched unrelated donor were CMV-infected. All but one CMV infections developed within 100 days after BMT, the median time being 32.5 days (range: 16-184; first and third quartiles 23.8, 47.5).

Sex, age, CMV antibody prevalence in donors and recipients and underlying diseases did not differ significantly between infected and non-infected patients. A strong association was demonstrated between antigenemia positivity and GVHD score ($p<0.001$) and mortality was also related to GVHD ($p = 0.038$).

No statistical association was demonstrable between CMV infection and mortality ($p=0.07$) and the elevated mortality rate in CMV positive patients (57.1%) is probably related to GVHD.

In the group of 14 patients who needed a second BMT the incidence of CMV infection was 35.7%.The infections occurred between 10 and 46 days post-transplantation (median, 31).

Discussion

In this study we evaluated the sensitivity and specificity of the antigenemia assay and the polymerase chain reaction for monitoring BMT recipients; moreover incidence of CMV infections was evaluated by antigenemia and analyzed for clinical and personal variables such as age, sex, underlying disease, donor compatibility and GVHD.

In a subset of 60 samples PCR detected all 20 infected patients with a sensitivity of 100% and a specificity of 100%, antigenemia failed to recognize one case (sensitivity 95%). The discordant PCR positive result preceded the onset of antigenemia by a week indicating that PCR is important in the diagnosis of the very early phase of CMV infection[17] although it may be too sensitive a marker in the clinical setting and its introduction in routine diagnostic protocols needs further evaluation.

The incidence of CMV in the group of patients receiving allogeneic BMT was 46.7% similar to that observed in similar studies.[18] The relevant result of our work is that the incidence of CMV infections is related to the severity of immunosuppression induced by the conditioning regimen, the treatment of marrow and GVHD prophylaxis. There is, therefore, no surprise about the lack of antigenemia among the patients submitted to an autologous BMT, and the increase of positive antigenemia when a donor different from a HLA matched sibling was used.

Rather impressive was the discovery of the higher incidence in matched unrelated donor (MUD) BMT (85.7%) than in haploidentical BMT (41.7%). From the theoretical point of view a matched transplant should assure a better immunologic reconstitution and a reduced risk of viral infections. As a matter of fact in our clinical experience there was no difference in GVHD severity in MUD and haploidentical transplants. We must consider that the so-called HLA match is an illusion outside the family of the patient; beyond the 6 HLA loci that result as being identical, there are other genetic markers that are unavoidably different between a patient and his MUD donor. In order to reduce the severity of GVHD these patients are submitted to a conditioning regimen and GVHD prophylaxis that are heavier than that used for a transplant from a matched sibling donor. In most cases anti-thymocytic globulin is given before the transplant.

Our patients submitted to a mismatched BMT received a conditioning regimen not different from the one received by MUD transplant recipients; GVHD prophylaxis did not include metothrexate together cyclosporin A, while MUD BMT protocols usually provide a double drug prophylaxis.

The treatment of donor's marrow in haploidentical transplants was performed with a technique that does not deplete T-lymphocytes: we use a functional T-cell depletion achieved by means of *in vivo* incubation with vincristine and methylprednisolone. Such a cocktail interferes with the activity of both Th1 and Th2 lymphocytes, without inducing apoptosis.[19] The viability of T-lymphocytes is assured and therefore the risk of late viral infections is, as demonstrated in our survey, not worse than that following a MUD transplant.

References

1. HG Prentice, E Gluckman, RL Powles, et al. Long- term survival in allogeneic bone marrow transplant recipients following acyclovir prophylaxis for CMV infection. Bone Marrow Transplant 1997; 19:129-33.
2. Griffiths PD Current management of cytomegalovirus disease.J Med Virol 1993; 1:106-11.
3. D Couriel, J Canosa, H Engler, et al. Early reactivation of cytomegalovirus and high risk of interstizial pneumonitis following T-depleted BMT for adults with hematological malignancies. Bone Marrow Transplant 1996; 18:347-53.
4. Y Takemoto, H Takatsuka, H Wada, et al. Evaluation of CMV/human herpes virus-6 positivity in bronchoalveolar lavage fluids as early detection of acute GVHD following BMT: evidence of a significant relationship. Bone Marrow Transplant 2000; 26:77-81.
5. AJ Huaringa, FJ Leyva, J Signes-Costa, et al. Bronchoalveolar lavage in the diagnosis of pulmonary complications of bone marrow transplant patients. Bone Marrow Transplant 2000; 25:975-9.
6. D Gor, C Sabin, HG Prentice, et al. Longitudinal fluctuations in cytomegalovirus load in bone marrow transplant patients: relationship between peak virus load, donor/recipient serostatus, acute GVHD and CMV disease. Bone Marrow Transplant 1998; 21: 597-605.
7. HG Prentice, P Kho. Clinical strategies for the management of Cytomegalovirus infection and disease in allogeneic bone marrow transplant. Bone Marrow Transplant 1998; 19:135-42.
8. G Gerna, M Zavattoni, E Percivalle, et al. Rising Levels of Human Cytomegalovirus (HCMV) Antigenemia during Initial Antiviral Treatment of Solid-Organ Transplant Recipients with Primary HCMV Infection. J Clin Microbiol 1998; 4:1113-6.
9. CM Machado, FL Dulley, LS Vilas Boas, et al. CMV pneumonia in allogeneic BMT recipients undergoing early treatment or pre-emptive ganciclovir therapy. Bone Marrow Transplant 2000; 26:413-7.
10. P Ljungman. Immune reconstitution and viral infections after stem cell transplantation. Bone Marrow Transplant 1998; 21 (Suppl. 2): S72-4.
11. M Boeckh, S Riddel, T Cunninggham, et al. Increased risk of late CMV infection and disease in allogeneic marrow transplant recipients after ganciclovir prophylaxis is due to a lack of CMV specific T-cell responses. Blood, 1996; 88 (Suppl. 1):302a.
12. CR Li, PD Greenberg, Mj Gilbert, et al. Recovery of

HLA-restricted cytomegalovirus (CMV)-specific T-cell responses after allogeneic bone marrow transplant: correlation with CMV disease and effect of ganciclovir prophylaxis. Blood, 1994; 83:1971-9.
13. CR de Medeiros, VA Moreira, R Pasquini. Cytomegalovirus as a cause of very late interstitial pneumonia after bone marrow transplantation. Bone Marrow Transplant 2000; 26:443-4.
14. H Sakuma, M Hosoya, H Kanno, et al. Risk of cytomegalovirus infection after peripheral blood stem cell transplantation. Bone Marrow Transplant 1997; 19:49-53.
15. Alejo Erice. Resistance of human cytomegalovirus to antiviral drugs. Clin Microbiol Rev 1999; 2:286-97.
16. GJ Demmler, GJ Buffone, CM Schimbor, et al. Detection of cytomegalovirus in urine from newborns by using polymerase chain reaction DNA amplification. J Infect Dis 1988; 158:1177-84.
17. AM Vlieger, GJ Boland, NM Jiwa, et al. Cytomegalovirus antigenemia assay or PCR can be used to monitor ganciclovir treatment in bone marrow transplant recipients. Bone Marrow Transplant 1992; 9:247-53.
18. H Einsele, G Ehninger, H Hebart, et al. Polymerase chain reaction monitoring reduces the incidence of cytomegalovirus disease and the duration and side effects of antiviral therapy after bone marrow transplantation. Blood 1995; 86:2815-20.
19. A Lanfranchi, M Andolina, K Tettoni, et al. Functional depletion of cells by vincristine and methylprednisolone as an in vitro model for the prevention of graft versus host disease. Haematologica 1992; 77:11-5.

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Artificial nutrition and bone marrow transplantation

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ABSTRACT

Parenteral nutrition has a central role in the supportive therapy of patients submitted to a BMT. A central catheter is mandatory for transfusions, antibiotic therapy and a proper nutrition. A good nutritional support contributes to maintain hydration, reduce lean body mass loss, increase patient comfort and improve survival in patients who can not eat or absorb for a prolonged period of time. After a BMT metabolic complications are frequent and require careful monitoring; in critical care patients, the major risks are electrolyte and glucose disturbances. Liver disease is a main metabolic complication of PN, but it can occur in any cancer patient due to therapy or to graft-versus-host disease. Its best prevention requires the avoidance of prolonged enteral fasting.

Key words: artificial nutrition, bone marrow transplantation

The optimal provision of calories and nitrogen is a challenging problem in patients undergoing bone marrow transplantation (BMT), which always results in a very negative nitrogen balance and in a greater loss of lean body mass (muscle) than fat mass or weight loss. Energy expenditure has been demonstrated to be 1.3 times the basal metabolic rate, so causing increased energy requirements, that can not be met in the patient because of anorexia, vomiting, and diarrhea. The negative nitrogen balance, the main feature of hypercatabolism, is associated with loss of muscle and also visceral proteins, and with a high risk of developing kwashiorkor: generalized edema, fatty liver infiltration, dermatitis, loss of muscle tone, and secondary immunodeficiency.[1]

In children with cancer, the increased metabolic rate is the complication of the disease, its consequences and their treatment, e.g. infections, anorexia, malabsorption, renal losses, mechanical gut problems related to alkaloids and other drugs, steroids, and contributes to the development of malnutrition. Many studies have, however, recently focused on the role of inflammatory cytokines as a specific risk factor. High levels of many cytokines have been demonstrated in patients in a critical condition. These levels correlate with the severity of the shock, multiple organ failure and also mortality. In cancer patients, cytokines determine anorexia and cachexia; some encouraging studies seem to indicate that monoclonal therapy against cytokines might be a new therapeutic choice for the future.[2]

Many studies have focused on the relationship between malnutrition and clinical outcome; in hospitalized patients malnutrition has been demonstrated to be associated with increased mortality, prolonged hospital stay, and increased cost. Weakness, compromised immunity, poor wound healing, and complications are more likely to occur in malnourished patients. In aging women undergoing hip replacement outcome is strongly related to the nutritional status, as it is in surgical or even in cancer patients: the better the nutritional status the better the outcome (mortality, morbidity, hospital stay, costs).[1,6] In children affected by malignancies malnutrition is associated with a poorer outcome, with a reduced tolerance to chemotherapy and, as a rule, with a higher susceptibility to infections.

In children, malnutrition reflects on growth pattern, but not only: specific deficits of some micronutrients (e.g. vitamins or trace elements) result in impairment of neurologic development, such as in case of iron, vitamin E and PP, and essential fatty acid (EFA) deficiencies. Other micronutrient deficiencies produce a clinical spectrum similar to that in adults. Growth impairment remains the rule, but there are also other complications that could interfere with malignancy treatment: zinc deficiency, a frequent consequence of diarrhea or intestinal losses, is associated with growth failure, greater susceptibility to infection , dermatitis, and anemia; copper deficiency leads to neutropenia, refractory anemia, osteoporosis and delayed bone age; selenium to heart failure.[2]

Although we can clearly demonstrate the relationship between malnutrition and outcome, the effect of nutritional therapy (artificial nutrition) is not so evident. Indeed, it is very difficult to demonstrate statistically, using a randomized clinical trial (RCT), the effect of one single variable (the nutritional status) on a complex disease. Some studies (RCTs) have demonstrated

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that short-term artificial nutrition does not decrease the complication rate in cancer patients receiving chemotherapy or radiation; by contrast, in patients given parenteral nutrition, there is an increase in infections, related to the central line. In bone marrow-transplanted patients, PN does not decrease treatment toxicity, infection rates, graft-versus-host disease, and only one RCT demonstrated a better long-term survival and a decreased tumor relapse rate. PN used as an adjuvant therapy, conferred no advantage to children with cancer if malnutrition had been treated before the specific therapy: that is, it is likely to have a better result if malnutrition is corrected. In one trial and in other uncontrolled studies, PN was associated with improved nutritional status (weight gain). In well-nourished children with cancer, the weight gain reflects increased deposition of fat rather than of lean body mass: a higher calorie intake in a well-nourished patient is associated with an increased fat deposition, as indeed expected. The results of the studies seem to indicate the absence of any real benefit of artificial nutrition on outcome, especially if there is no malnutrition.[1,6]

Nevertheless, it is reasonable that nutritional support contributes to maintaining hydration, reducing lean body mass loss, increasing patient comfort and improving survival in patients who can not eat or absorb for a prolonged period of time, in patients with malnutrition or at risk of it. The benefit in non-compromised patients is less evident, if disease outcome parameters are the endpoints. However, the aim of nutritional support should be the avoidance or treatment of malnutrition and not the treatment of the underlying disease; from this point of view, it is likely that nutritional support is beneficial in critically ill patients undergoing a hyercatabolic procedure such as bone marrow transplantation. Nutritional support must improve the nutritional status, while keeping technical and metabolic complications related to the line care to a minimum.

Artificial nutrition can be delivered enterally (enteral nutrition, EN) or through a venous central line (parenteral nutrition, PN). The relative benefit of EN versus PN has been widely discussed but so far no data have convincingly shown that EN is better than PN. Enteral nutrition is supposed to be safer, cheaper, more physiologic, to promote normal gastrointestinal function, to prevent bacterial translocation and to be associated with better outcomes than parenteral nutrition, but so far no study has been conclusive . In a recent meta-analysis on critically ill patients, PN was not associated with a worse outcome, and many other studies, performed in different patients, failed to show a real benefit of enteral nutrition, so the choice between enteral and parenteral nutrition seems to be based on team experience, patient's feeding tolerance, presence of intestinal obstruction, and need for prolonged intravenous therapy. However, if there is an indication for artificial support, enteral nutrition should be considered as the first choice.[1,2,5]

In patients undergoing bone marrow transplantation, the complications related to chemotherapy and radiation could represent a formal indication for parenteral nutritional support, because anorexia, vomiting, and enteritis can interfere with feeding tolerance. The prolonged intravenous therapies require, especially in children, the presence of a central venous line which is also useful for nutrition. PN is a safe technique for nutrition, but the rate of complications must be considered. Technical complications are sepsis and line/venous obstruction; the metabolic complications include hyper- and hypoglycemia, electrolyte disturbances, liver disease, and osteopenia.[1,2]

The most frequent complications are those related to the infusion line, especially infections; handwashing and aseptic technique in the management of the line are the basic principles of infection control. Large studies showed that not only is the aseptic management of the line important to minimize the risk of infection but also its correct placement. Controlled studies have demonstrated the benefit of placement in the operating room, using good barrier precautions, i.e. masks, cap, sterile gloves, gown and a large drape to cover the insertion site. With such a protocol, the colonization rates dropped from 1.0 per 1,000 days to 0.3 per 1000 days. The catheter-related sepsis rate was 6.3 times higher in untreated groups. Catheter tunnelling seems to play a role in preventing sepsis but not such an important and relevant one as the role of the nutritional team. Many studies have strongly suggested that the presence of a motivated nursing team using the correct aseptic technique in managing the line is the best prevention of catheter-related sepsis; far less important are the new catheters, silver-chelated or antibiotic impregnated lines. In contrast, the use of the new needleless system for the closure of the line offers a significant advantage in terms of nursing and patient safety: the needleless devices permit connection to the line without opening the catheter, thus reducing the risk of infection, and also of blood reflux (the system is always closed). The concerns about the increased risk of infection arose from the first experiences, but further studies have clearly indicated a drop in infection rates if the device is changed at least every 72 hours.[7]

Metabolic complications are frequent and require careful monitoring; in critical care

patients, the major risks are electrolyte and glucose disturbances, sometimes also related to complications of the underlying disease. In long-term parenterally-fed patients, the risk of developing micro- and macronutrient deficiencies must be taken into account, because of the difficulty in providing an adequate and *complete* PN-admixture. In bone marrow transplanted patients, the major deficiency risk is related to the limited administration of lipids, often not given because of sespis, and pharmacologic incompatibility with other therapies. In children EFA-deficiency can be detected after 1 week without a EFA intake that is at least 1% of the total daily calorie intake; linoleic and linolenic acids are not synthesized by humans and are both considered *essential*; they are the precursors of arachidonic acid (from linoleic) and of docosahexaenoic and eicosapentaenoic acids (from linoleic acid), which are involved in central nervous system development. These essential fatty acids are necessary for growth, skin and hair integrity, regulation of cholesterol metabolism, decreased platelet aggregation, and lipotropic activity. The parenteral lipid emulsions are represented by long chain fatty acids (LCT), by a mixture of long and medium chain triglycerides (LCT-MCT), and by the more recent olive oil derivates. Their use is often limited because of concern about the effects on immune and reticulo-endothelial systems, by the mediation of several systems, including increasing E2 production, decreasing T helper / T suppressor ratio, inhibiting neutrophil migration, chemotaxis, endotoxin clearance, and complement synthesis and depressed natural killer and lymphokine activated killer activity by blockage of interleukin-2 binding to its receptor.[2,4]

PN solution means a *complete* admixture of at least glucose, nitrogen, salts, minerals; the provision of glucose and salts is not parenteral nutrition, but hydration support. Lipids need not be given daily, but they must be given one/two days a week in order to cover basal needs; zinc and copper supplementation will be required if PN lasts for more than 2 week, while complete provision of all known trace elements is strongly indicated for PN of longer duration, in order to avoid a deficiency syndrome. The complexity of a PN solution grows with the duration of the artificial support, particularly if nutrition is exclusively being given by the pare-nteral route, i.e. in patients who cannot eat even small amounts of food. The definition of parenteral intakes is based on the metabolic status of the patient (presence of hypercatabolism and/or malnutrition) and on theoretical calorie and nitrogen needs; the intakes must be closely monitored to avoid the most frequent complications. In critically ill patients, a constant infusion rate generally allows better metabolic tolerance, especially for lipids, which must be delivered over at least 8-12 hours in order to reduce the risk of hypertriglyceridemia which can occur if the infusion rate exceeds clearance capacity (the infusion rate should be a maximum of 0.17 g/kg/h).[2]

The use of glutamine, a conditionally-essential aminoacid, has been claimed to be important in decreasing mortality and morbidity in patients undergoing bone marrow transplantations, as well as in other clinical conditions. Its role in muscle function, in nitrogen transport to the cells, as a primary fuel for enterocytes, and in preserving the integrity of mucosal structure and function of the intestine, appear to be crucial and many clinical trials have been performed in order to demonstrate its effect, given either parenterally or orally. There is, however, no clear evidence so far that glutamine is useful in improving outcome in these patients and its use needs further investigation.[3]

Liver disease is a main metabolic complication of PN, but can occur in any cancer patient due to therapy or to graft-versus-host disease. Its best prevention is avoidance of prolonged enteral fasting, infections, and surgery. As far as concerns the PN admixture, the lower the calorie content, the lower the probability of developing liver disease: as a rule, calorie intake should not exceed the (theoretical) needs, so as to reduce the risk of hepatic fat deposition. The evolution toward severe liver damage is more frequent in low-birth weight neonates and in children on long-term parenteral nutrition (months, years). This having been said, in any given situation it is more likely that liver involvement is related to the underlying disease than to well-conducted parenteral nutrition.[1,2,6]

In conclusion, even in the absence of RCT clearly demonstrating its efficacy on disease outcome, artificial nutritional support seems to be useful in bone marrow transplanted patients in order to avoid or correct malnutrition, which is a frequent and multifactorial complication of the procedure. An experienced team (nurses, surgeon, pediatrician, pharmacist) is the best preventive measure against technical and metabolic complications.

References

1. A.S.P.E.N. Board of Directors. Guidelines for the use of parenteral and enteral nutrition in adult and pediatric patients. JPEN 1993; 17:1SA- 38SA.
2. Candusso M. Nutrizione del bambino ospedalizzato: principi e pratica. M&B 2000; 19:289-94.
3. Demirer S, Aydintug S, Ustun C, et al. Comparison of the efficacy of medium chain triglycerides with long

chain triglycerides in total parenteral nutrition in patients with hematologic malignancies undergoing peripheral blood steam cell transplantation. Clin Nutr 2000; 19:253-8.
4. Klein S, Kinney J, Jeejeebhoy K et al. Nutrition support in clinical practice: review of published data and recommendations for future research directions. JPEN 1997; 21:133-56.
5. Lipman T. Grains or veins: is enteral nutrition really better than parenteral nutrition? A look at the evidence. JPEN 1998: 22:167-82.
6. Mauer AM, Burgess JB, Donaldson S, et al. Special nutritional needs of children with malignancies: a review. JPEN 1990; 14:315-24.
7. Schloerb PR, Skikne B. Oral and parenteral glutamine in bone marrow transplantation: a randomized, double-blind study. JPEN 1999; 23:117-22.

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The central venous catheter in a bone marrow transplant unit: an unresolved problem

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ABSTRACT

Bone marrow transplantation (BMT) is feasible with a bearable risk and discomfort for patients only if good venous access is provided. Therefore a major task for nurses of a BMT unit is management of a patient's central venous catheter. There is not general agreement about the procedure of handling a CVC and infection prophylaxis. We collected data from some Italian BMT and hematology units by means of a questionnaire. The responses to this questionnaire were not comparable except for some particulars. Each center has its own ritual procedure; even the use of sterile gloves while handling the most dangerous connections of the catheter is not the rule everywhere. It is noteworthy that only a minority of physicians are able to handle a catheter correctly.

Key words: central venous catheter, bone marrow transplantation, infection

A central venous catheter (CVC) has long been accepted as the most suitable indwelling intravenous system for patients in need of a bone marrow transplantation (BMT).

Its use and efficiency have been widely demonstrated in daily and emergency situations since it is always available and can be used to introduce large quantity of fluids into a patient, including simultaneous mixtures of more than one type of drugs, blood and all its components, blood derivatives, and partial and total parenteral nutrition, even with an elevated concentration of glucose.

For all these reasons the CVC is considered an indispensable instrument. Nevertheless it represents an opening to external micro-organisms allowing direct access to the blood circulation and causing severe, at times fatal, infections.

The CVC, besides being a "door of access" to the outside can also be colonized by some internal micro-organisms, forming an inexhaustible source of infection; therefore the only remedy, depending on the micro-organism involved, is to put an end to this situation, by removing of the CVC.

For this reason, the CVC, which is still a cause of mortality in patients exposed to aggressive infective agents, is object of study and research and indeed the handling of this irreplaceable (during aplasia) instrument remains an unresolved problem.

When an infection, possibly due to the CVC, occurs, the nurses feel very involved, because they are the only staff assigned to work and handle the CVC, according to their own protocols and /or procedures consolidated in time to avoid contamination.

Therefore it is useful to talk more about this aspect of BMT.

Sharing opinions is not enough: we should concentrate on a retrospective research, reviewing our own methods, in order to organize all the specific data collected better and to obtain an operative protocol to be used in all the BMT centers in our country. Only in this way will we be able to begin realistic research with many available data on a certain type of patient. Such work has been started and concluded in the Associazione Italiana Emato Oncologia Pediatrica (AIEOP) Infermieri and includes the heparinization and care of the insertion site of the CVC.

To present our experience here would have been interesting, but unfortunately there was not enough time for retrospective research,complicated by the difficulty in reading clinical files. Besides it would be impossible to gather significant data to establish the correct method of work comparing our protocols with those working with the same type of patients and instruments.

The questionnaire

We forwarded a questionnaire to nurses in 84 BMT centers in Italy in order to gather information on the new approaches to CVC manaethat that could supply a general outlook on the current situation.

Of the 84 centres we consulted, only 26 replied, of which 4 responses were invalid

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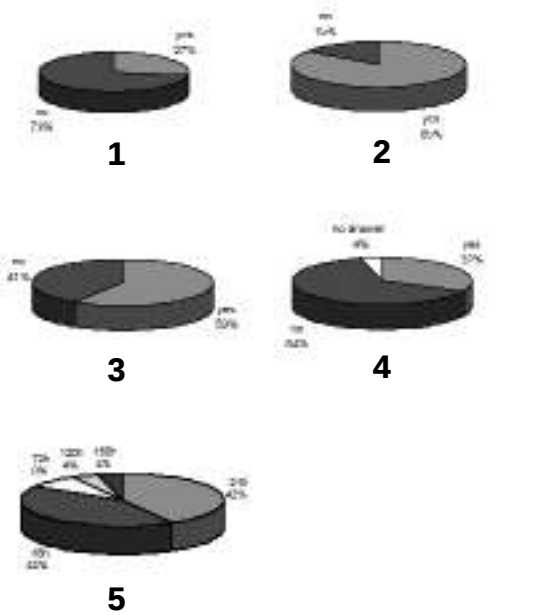


Figure 1. Answers to the questionnaires.
Figure 2. Unevaluable questionnaires.
Figure 3. Use of a laminar flow hood.
Figure 4. Use of antibacterial filters.
Figure 5. Change of circuit infusion (in hours).

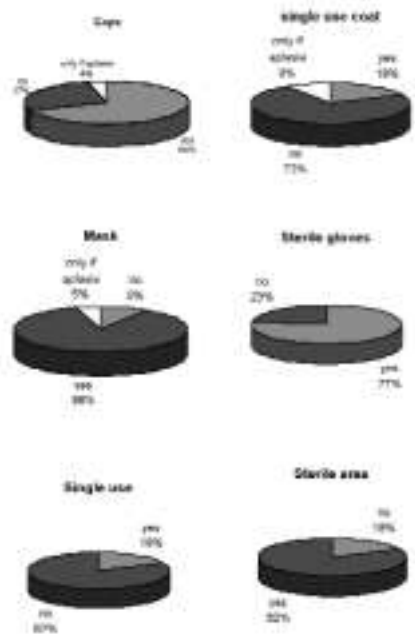


Figure 6. The measure of prevention for the CVC medication.

because not completed.

The lack of replies could have been caused by the fact that many centers, in particular those that deal mainly with adult patients, use CVC that are not tunneled, while our research covered only the Broviac, Hickman, Groshong and Port-a-Cath varieties of CVC.

The answers obtained were varied and of difficult comparison because the use of the instruments in the various centrers is different: for examples the positions of the antibacterial filters along the line of infusion or use of a laminar flow hood. We found differences in management of the protection of the collection joints which could be loose, wrapped in gauze or in a system of dried and sterilized disposable protection or soaked in iodiopovidon.

These instruments and methods are not used in standardized way in the same patient, but change according to neutrophil count.Thus the use of a mask and cap and a longer length of time between changes of the infusion line are determined by the patient's neutrophil count.

The only datum that unites all the centers is the method of caring for the insertion site of the CVC (82%). What differentiated was the use of more anti-infective solutions, the interval between the medications and the use of topical therapy even without sign of infection. The method of infection prevention was considered good by 86% of the centers, sufficient by 10% and exagerated by 4%!

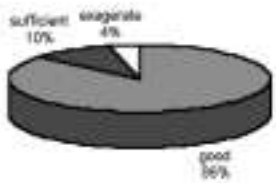
Discussion

What seemed to us an efficient and original idea was demolished by the results obtained, although this does not mean that we have abandoned our research for the future.

At this point another questionnaire would have been useful to evaluate the number of infections correlated with the CVC, occurring in each center, but that would have required more commitment from my colleagues. It seems unlikely that any survey will be able to demonstrate that in terms of prevalence of infection one system is superior to others. Too many factors are involved in causing infections: conditioning regimen, type of transplant (allo or auto), previous chemotherapy and mucosal damages.

The results of the questionnaire have made us think about some of aspects of CVC handling, with regards to the antiseptic techniques, used to prevent infections.

We ask ourselves why so much care is taken of the insertion point, protected so perfectly with antiseptic technique, but less attention is dedicated to the direct handling of the CVC. For example some colleagues change the infusion



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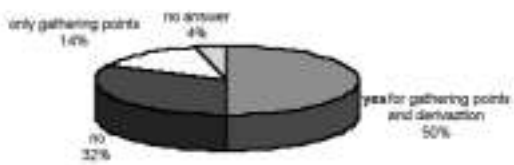

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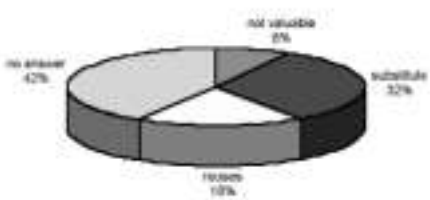

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Figure 7. Efficiency of working method.
Figure 8. Protection at collection points of CVC and derivations.
Figure 9. Reutilization of the protective points of the joints and derivations.

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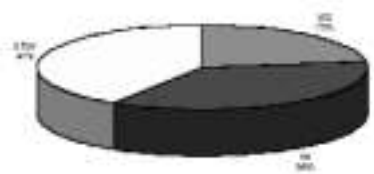

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Figure 10. Elaboration of protocol.
Figure 11. The knowledge of doctors on the methods and handling of CVC.

lines without sterilized gloves and use the derivation circuit to administer therapy without any protection.

From what do we deduce that for one operation we need to apply all the rules of antiseptic care, and for another it is sufficient to wash our hands ? Why does a CVC need more care because the patient is neutropenic, when there are no guarancies that the neutropenia will manifest again? In our opinion it is a curious attitude that makes one act with extreme caution on an area of insertion which is cicatrized, one in which one or more mechanical barriers (cuffs in Dacron) are present and yet not worry to the same degree when connecting or injecting directly into the blood circulation interposing, only hand washing as an infection barrier.

We ask ourselves whether this difference is reasonable. Maybe the same importance given to the maintenance of an intravenous short-term catheter is widened to a CVC, forgetting that the tunneled CVC was designed to remain in place for long periods of time, and for this reason should be protected from possible infections. Or maybe the patient submitted to BMT has been compared to the one in intensive care, undervaluing the diversity of immune system function in these two groups of patients, disregarding that, even when there is a recovery from neutropenia, the immune system in the BMT patient remains extremely oversensitive to infective agents.

Not by chance the majority of protocols in use in some centers were produced together with anesthetists, heart surgeons, or surgeons who use an indwelling CVC, who have many years of experience in the use of CVC but not in the specific context of BMT.

We think it is proper to consider the experience of others but that the choice of a technical model of assistance is based on the demand that a pathology requires, and must be discussed with the experts who know the consequences of an infection of the CVC.

One item of the questionnaire addressed the knowledge of the method in use by doctors of the BMT centers: only 23% gave an affirmative answer.

The good result of a BMT is not exclusively linked to the efficiency of the therapeutic protocol, but also to the prevention of infections, a sphere in which nurses assume a significant role.

References

1. Clarke DE, Raffin TA. Infectious complications of indwelling long-term central venous catheters. Chest 1990; 97:966-72.
2. Goldmann DA, Pier GB Pathogenesis of infection related to intravascular catheterization. Clin Microbiol Rev 1993; 6:176-92.
3. Maki DG Pathogenesis, prevention, and management of infections due to intravascular devices used for infusion therapy. In Bisno AL, Waldvogel FA (Eds): Infections associated with indwelling medical devices. Washington, DC. American Society for Microbiology 1989; pp:161-77.
4. Linee guida per la prevenzione delle infezioni associate a dispositivi intravascolari: Centers for Disease Control and Prevention. Giornale Italiano delle Infezioni Ospedaliere, vol. 4,n.1, Gennaio-Marzo 1997.
5. Daghistani D, Horn M, Rodriguez Z, Schoenike S, Toledano S. Prevention of Indwelling Central Venous Catheter Sepsis. Med Pediatr Oncol 1996; 26:405-8.

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High dose therapy and autologous hematopoietic stem cell transplantation in poor risk solid tumors of childhood

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ABSTRACT

In the last two decades autologous hematopoietic stem cell transplantation (HSCT) has been increasingly used in the treatment of several poor risk solid tumors of childhood. Examples are recurrent or resistant cancers, metastatic presentation at diagnosis, incomplete surgical resection, unfavorable histologic and biological features.
Results from the Children's Cancer Group randomized trial confirm the data from retrospective studies which reported the superiority of HSCT over standard chemotherapy for neuroblastoma. Several retrospective analyses support the use of HSCT in Ewing's sarcoma and in some brain tumors. No evidence of utility has been reported for rhabdomyosarcoma . The most widely utilized source of stem cells is peripheral blood, while there are conflicting data regarding the use of total body irradiation and purging of stem cells.

Key words autologous hematopoietic stem cell transplantation, children, solid tumors.

The prognosis of children with advanced, recurrent or refractory solid tumors is poor. Examples of advanced cancer in children include patients over 1 year old with disseminated neuroblastoma or this tumor with unfavorable biological markers and patients with metastatic Ewing's sarcoma.[1,2]

Over the last two decades the use of dose-intensive chemo/radio therapy allowed by autologous hematopoietic stem cell transplantation (HSCT) has improved the outcome of these patients.[3]

The aim of this paper is to present a short overview of some significant clinical data reported in the literature for the most frequent childhood cancers and to illustrate some points actually under discussion such as stem cell source, minimum threshold of $CD34^+$ cells to infuse, sequential HSCT and tumoral contamination of inoculum.

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Neuroblastoma

Neuroblastoma (NB) is the most common extracranial solid tumor of childhood. In nearly 60% of the cases it develops with bad prognostic characteristics, such as age more than 1 year, metastatic dissemination, MYCN oncogene amplification and unfavorable histopathology.[1] Conventional chemotherapy is able to cure fewer than 10% of these patients.[4]

A number of retrospective studies[5-7] carried out in the last 10-15 years showed that, using myeloablative therapy and autologous bone marrow transplantation, a long-term disease-free survival (DFS) could be obtained for 20-40% of children.

The summary of 15 years of European experience[6] regarding 549 patients more than 1 year at diagnosis of stage IV NB, receiving HSCT as consolidation of primary treatment (complete remission-CR, very good partial remission-VGPR and partial remission-PR), showed a 5-year event-free survival (EFS) of 26%.

Significant univariate factors predicting unfavorable outcome were: positive bone marrow (BM) involvement at diagnosis ($p = 0.53$), persisting BM involvement ($p = 0.03$), and persisting positive skeletal disease at ^{99}TC and I^{131}meta-iodobenzylguanidine (MIBG) scan ($p=0.004$). Prolonged treatment duration before HSCT ($p=0.002$) and a double HSCT ($p=0.05$), had a favorable influence on prognosis. Response status before HSCT (CR versus VGPR versus PR) had no influence. This unexpected absence of difference in EFS could be explained by the non homogenous evaluation of remission status, probably due to the fact that during the 15 years of the study more sensitive techniques have become available.

In the multivariate analysis using a Cox proportional hazards regression model, persisting skeletal lesions ($p=0.004$) and persisting BM involvement before HSCT ($p=0.03$) were identified as independent risk factors influencing EFS. The toxic death rate, before and beyond day +100 either in single or in double HSCT, was 17% , a very high incidence than in recent years has been greatly reduced by better supportive

therapy including colony-stimulating factors and infusion of large quantities of peripheral blood progenitor cells.

In the two biggest reviews published by the EBMT group[5,6] no differences in EFS were reported between patients receiving purged and non-purged stem cell transplantations.

In a recent multivariate analysis of 218 patients treated in a single institution[7] between 1980 and 1996, the probability of DFS at 5 years was 29%, toxic death occurred overall in 26 children (12%), although this decreased from 25% before 1990 to 5% after 1990. The source of stem cells was allogeneic in 12 cases, from peripheral blood in 5 cases, purged autologous BM in 172 cases and non-purged autologous BM in the remaining children.

All preparative regimens contained high dose melphalan and approximately 50% of patients also received busulfan.

In the univariate analysis, age over two years (p=0.033) and preparative regimens without busulfan or melphalan had a strongly unfavorable prognostic value. As in the analysis of Ladenstein *et al.*, disease status at transplant was not significantly associated with prognosis, although it came close to being so (p=0.053).

The EBMT Solid Tumors Working Party booklet, distributed during the last EBMT Meeting (Innsbruck March 2000),[8] listed 1,935 patients, grafted between 1985 and 1999 with different disease statuses (relapse/progression = 213, consolidation = 1531, stable disease = 37, unknown = 154). In the 1,489 assessable children, in whom HSCT was performed as consolidation after first line therapy, the overall survival was around 35%, and the EFS was 31% with a median follow-up of 51 months. In the 633 patients grafted in first remission the progression-free survival (PFS) was around 40% with a median follow-up of 46 months. Other reported information was a significant advantage (p= 0.001) for patients younger than two years at diagnosis, and that the toxic death rate has decreased from the 19% before 1986 to the actual 8%.

The recent paper published by Pession *et al.*[9] gives us useful information about activity and results in Italy. The EFS at 5 years for the whole group of 273 patients with NB after HSCT was 30.4%, increased to 37.8% for children in 1st CR – VGPR. For those children with less responsive tumors, the EFS at 5 years decreased to 24%. The transplant-related mortality (TRM) decreased from 11% in 1988 to less than 5% in 1998 ($p < 0.05$) irrespectively of disease status at transplantation.

In October 1999 the results of the prospective, randomized Children's Cancer Group study were published.[10] This study compared a combination of myeloablative chemotherapy, TBI and purged autologous BMT with intensive non-myeloablative chemotherapy; a second randomization was performed to determine whether subsequent 13-cis-retinoic acid treatment could further improve EFS.

The results are the following: a) the 3 years EFS rate in 129 patients who underwent transplantation was 43±6% compared with a rate of 27±5% for patients who received chemotherapy; b) the estimated EFS 3 years after the 2nd randomization for patients receiving 13-cis-retinoic acid after BMT was 55±10% as compared with 18±6% for those treated with chemotherapy only. The 3 years EFS rate of patients receiving 13 -cis-retinoic acid after either treatment was better than the rate for those assigned to no further therapy (46±6% vs 29±5%, p = 0.027).

Univariate analysis revealed a series of adverse prognostic factors: stage IV disease, amplification of MYCN oncogene, unfavorable histopathologic findings, high levels of ferritin and partial response to initial chemotherapy.

Only for stage IV patients were bone involvement and more than 100 tumor cells per 105 nucleated cells at diagnosis adverse prognostic factors.

The conclusions of the paper are that "*these therapeutic approaches should form the basis for the treatment of patients with high risk NB*".

In conclusion data from retrospective and prospective randomized studies suggest that patients with poor risk NB gain a significant advantage from high dose therapy and autologous stem cells rescue over those receiving chemotherapy.

The prognostic influence of disease status at graft is controversial, but at least two recent papers[7,10] report data in favor of the so-called *good responders*.

The most effective preparative regimen has not been yet identified, and randomized studies comparing total body irradiation (TBI) versus non-TBI use have not been reported. The absence of clear clinical data about the role of TBI in pediatric solid tumors and the knowledge of its potential long-term effects have led, especially in European Countries, to an increased use of regimens without TBI, often containing busulfan and/or melphalan.

Growing importance is attributed to the role of therapeutic dosages of MIBG administered in addition to megatherapy[11] or immediately after hematologic recovery, for the most part in patients with residual disease. This approach is to be compared, in matched groups of patients, with local external irradiation.

The risk of tumoral contamination of the autol-

ogous stem cells causing recurrence of disease, the importance of sensitive and specific assays (e.g. immunocytology and reverse transcription-polymerase chain reactions) and the role of various methods of selection will be discussed briefly in the chapter dedicated to general aspects.

There are some reports about stem cell rescue with selected $CD34^+$ cells.[12,13] In 1997, Handgretinger *et al.* published their results about the treatment of 20 children with NB, 15 of whom had received positively selected $CD34^+$ peripheral progenitor cells. A mean log depletion of tumor cells of 1.41 was obtained with prompt (median 12 days, range 10-16 ANC) neutrophil recovery (greater than 0.5×10^9/L) and prolonged platelet recovery (median 30 days, range 16-150 to transfusional independence). No data about long-term side-effects or EFS are reported. Kanold *et al.*[13] reported a series of 23 patients in whom successful neutrophil reconstitution but delayed platelet recovery (59 days, range 22-259 to more than 50×10^9/L) was seen. Major concerns are the persistently low in $CD4^+$ lymphocyte levels and the high incidence of serious late infections, especially the occurrence of 2 EBV-lymphoproliferative diseases.

Ewing's sarcoma

Metastatic disease at diagnosis, associated with large tumor size and pelvic site are the most significant adverse prognostic factors for patients suffering from Ewing's sarcoma; in this group the EFS at two years is < 30%.[14]

Two analyses based on the EBMT experience were conducted. The first on 210 patients not in CR at the time of HSCT, the majority of whom had previous progressive disease, showed an overall response rate of 53% (CR obtained in 27%).[15] In the second trial, performed in 63 children in first or second CR, the DFS at five years was 21% and 32%, respectively, the non-TBI regimens being slightly more successful than the TBI-containing regimens, and the association of busulfan, melphalan seeming to produce the best DFS (51%).[16]

Remarkable results (DFS at 6 years,45%) were obtained by the CESS group in a small population of 17 patients using the association TBI, melphalan, etoposide ± carboplatin.[17] The same group suggested the usefulness of IL-2 immunotherapy after transplantation; the difference in DFS in children receiving or not receiving IL-2 is significant (52% versus 22%, $p < 0.05$).[18]

The challenge in the future will be a better definition of groups of patients, up to now all listed togheter as *poor risk patients* (for example those with marrow spread, multiple bone localizations, recurrent or resistant disease, excluding probably children with a single lung metastasis or late and isolated relapse) in which HSCT can be really advantageous. The immunologic characteristics of Ewing's tumor and the consequent possibility of targeted immunotherapy are fields to be explored.

Rhabdomyosarcoma

In the last ten years the reasons for performing HSCT in children with rhabdomyosarcoma (RMS) were the presence of metastasis and relapse after a primary response. Since 1989 the *European Intergroup Study* has designed two consecutive protocols for the treatment of patients with metastatic disease at diagnosis. The first study was based on a six-drug regimen without HSCT, while the second trial was carried out with HSCT in children in clinical CR after the second cycle of chemotherapy The results can be summarized as follows:[19] a) the achievement of CR after the second cycle is crucial with respect to the 3-year EFS for both groups (27.8% versus 10.8%, $p= 0.0001$); and b) the 3-year EFS rates were 29.7% for HSCT group and 19.2% for non HSCT group ($p= 0.3$).

The conclusions of this study as those of Koscielniak *et al*.,[20] are not in favor of routine use of HSCT in RMS and emphasize the need of controlled clinical trials.

Medulloblastoma and infants with brain tumors

The prognosis of a consistent number of children with newly diagnosed malignant brain tumors, especially for those with some unfavorable histologic subtypes, in the absence of radical surgical resection and with metastatic presentation remains poor despite surgery, irradiation and conventional chemotherapy. Similarly patients whose tumor recurs despite initial therapy continue to experience a dismal outlook with these conventional strategies of treatment. In an attempt to improve the outlook, strategies utilizing high-dose chemotherapy with autologous stem cell rescue have been developed. These studies, conducted initially in patients with recurrent tumors, were then extended to very young children with various malignant brain tumors at diagnosis in an attempt to avoid irradiation to the brain.[21]

In several reports[22-24] patients with recurrent medulloblastoma, for which long-term survivors are rarely described, have benefited from HSCT. In the report of the Duke University experience concerning 49 patients with recurrent brain tumors[23] an important message is that 4/6 cases with local recurrence are disease-free survivors, while none of the 12 with metastatic relapse were event-free survivors. In the Sloan-Kettering study[24] the EFS at 3 years of 23 patients treated with the association carbo-

platin-thiotepa-etoposide is 34%.

Since July 1997 in our Institution we have been applying a prospective protocol for children with the above mentioned characteristics. The four courses of induction therapy (phase A) consisted of methotrexate 8 g/m^2 + VCR 1.4 mg/m^2, etoposide 2.4 g/m^2, cyclophosphamide 4 g/m^2 + VCR 1.4 mg/m^2, carboplatin 800 mg/m^2 + VCR 1.4 mg/m^2. PBPC mobilization and collections were performed with rh-G-CSF stimulus in the recovery period after the 2nd and/or the 3rd cycle. Responding patients underwent two consecutive HSCT (carboplatin 1500 mg/m^2 + etoposide 500-600 mg/m^2 followed at recovery by thiotepa 30 mg/kg + melphalan 4 mg/kg) with PBPC reinfusion (phase B). Radiotherapy (phase C) was administered in case of residual disease and in patients older than 3 years. So far the double PBPC transplant has been performed in 15 cases. Our preliminary observations are that: 1) phase A has a good reductive capacity (72% of complete + partial remission) and 2) induces mobilization and collection of a large number of CD34$^+$ cells, in the majority of the patients with only one leukoapheresis, 3) the first high-dose course, considering the low incidence of toxicity, could be administered in the outpatient setting, 4) the hematologic and mucosal toxicity of the 2nd transplant is higher but acceptable. The role and modality of radiotherapy, as concerns its impact on long-term disease-free survival and side effects, need a longer period of observation.[25]

General aspects of HSCT in solid tumours of childhood

Stem cell source

In the last ten years peripheral blood progenitor cells have replaced bone marrow[8,9,26,27] as the source of stem cells because of faster engraftment, lower rate of complications, lesser supportive care needed and finally shorter duration of hospital stay.

The minimum number of CD34$^+$ cells to be infused is still controversial. Several authors[28,29] report that above a level of 3-5$\times$10^6/kg, sustained and trilineage engraftment is achieved. Leukaphereses are usually performed in the recovery phase after chemotherapy and G-CSF stimulus. A good yield of PBPC is obtained in patients without bone marrow involvement, in those receiving few chemotherapy cycles and without previous radiotherapy.[30]

Double high dose therapy

The availability of large numbers of CD34$^+$ cells, collected from the peripheral blood, has allowed the sequential administration of various combinations of high-dose regimens with and without total body irradiation. In the majority of the cases the drugs were alkylating agents, for which the steep dose-response curve is well known, mostly used in responding patients after a previous recurrent disease,[31] or in phase II studies for newly diagnosed poor-risk neuroblastoma.[32] The results are encouraging (EFS at 3 years 35% and 58%, respectively) and the toxicity appears to be acceptable (toxic death rate 16% and 8%, respectively).

The risk of tumoral contamination of the autologous stem cells

There are several reports, especially in neuroblastoma (NB) and Ewing's sarcoma, showing the presence and clonogenicity of tumoral cells in autologous BM and PBPC.[33,34] Although the reinfusion of active neoplastic cells can not be considered the only cause of relapse, a sensitive and specific assay for detection of malignant cells is, nowadays, mandatory. The methods to reduce the risk of reinfusing malignant cells to a minimum are effective *in vivo* purging by induction treatment before stem cell harvest, the evaluation of all stem cells used for HSCT, the use of PBPC (less frequently contaminated), *ex vivo* purging by immunomagnetic methods, monoclonal antibodies, and positive and/or negative CD34$^+$ selection.

Conclusions

In the last two decades HSCT has been increasingly used in the treatment of poor risk solid tumors in children. However some issues remain open to debate and need to be clarified, such as the comparison between HSCT and high-dose non-myeloablative therapy, the role of stem cell purging, the use of TBI and of tandem transplantation, post-HSCT immunotherapy and/or with drugs inducing differentiation. Due to the relative rarity of childhood cancers the only way to clarify so these issues will be cooperative and probably multinational trials.

References

1. Matthay KK. Neuroblastoma: biology and therapy. Oncology 1997; 11: 1857-66, 1869-72, 1875.
2. Grier HE. The Ewing family of tumours: Ewing's sarcoma and primitive neuroectodermal tumors. Pediatr Clin North Am 1997; 44:991-1004.
3. Seeger RC, Reynolds CP. Hematopoietic cell transplantation for neuroblastomas. In Thomas ED, Blume KG, Forman SJ, eds. Hematopoietic Cell Transplantation – Second edition, Blackwell Science, Inc, 1999. p. 1071-83.
4. Philip T, Zucker JM, Bernard JL, et al. Improved survival at 2 and 5 years in the LMCE1 unselected group of 72 children with stage IV neuroblastoma older than 1 year of age at diagnosis: is cure possible in a small

subgroup? J Clin Oncol 1991; 9:1037-44.
5. Philip T, Ladenstein R, Lasset C, et al. 1070 myeloablative megatherapy procedures followed by stem cell rescue for neuroblastoma: 17 years of European experience and conclusions. Eur J Cancer 1997; 33:2130-5.
6. Ladenstein R, Philip T, Lasset C, et al. Multivariate analysis of risk factors in stage 4 neuroblastoma patients over the age of one year treated with megatherapy and stem-cell transplantation: a report from the European Bone Marrow Transplantation Solid Tumor Registry. J ClinOncol 1998; 16:953-65.
7. Hartmann O, Valteau-Couanet D, Vassal G, et al. Prognostic factors in metastatic neuroblastoma in patients over 1 year of age treated with high-dose chemotherapy and stem cell transplantation: a multivariate analysis in 218 patients treated in a single institution. Bone Marrow Transplant 1999; 23:789-95.
8. EBMT Solid Tumours Working Party, Booklet 1999.
9. Pession A, Rondelli R, Paolucci P, et al. Hematopoietic stem cell transplantation in childhood: report form the bone marrow transplantation group of the Associazione Italiana Ematologia Oncologia Pediatrica (AIEOP). Haematologica 2000; 85:638-46.
10. Matthay KK, Villablanca JG, Seeger RC, et al. Treatment of high-risk neuroblastoma with intensive chemotherapy, radiotherapy, autologous bone marrow transplantation and 13-cis-retinoic acid. N Engl J Med 1999; 341:1165-73.
11. Klingebiel T, Bader P, Bares R, et al. Treatment of neuroblastoma stage 4 with 131I meta-iodo-benzylguanidine, high dose chemotherapy and immunotherapy. A pilot study. Eur J Cancer 1998; 34:1398-402.
12. Handgretinger R, Greil J, Schurmann U, et al. Positive selection and transplantation of peripheral CD 34+ progenitor cells: feasibility and purging efficacy in pediatric patients with neuroblastoma. J Hematother 1997; 6:235-42.
13. Kanold J, Yakouben K, Tchirkov A, et al. Long-term results of CD 34+ cell transplantation in children with neuroblastoma. Med Pediatr Oncol 2000; 35:1-7.
14. Cangir A, Vietti TJ, Gehan EA, et al. Ewing's sarcoma metastatic at diagnosis. Result and comparison of two intergroup Ewing's sarcoma studies. Cancer 1990; 66:887-93.
15. Ladenstein R, Hartmann O, Pinkerton R. The impact of megatherapy (MGT) followed by stem cell reinfusion (SCR) in Ewing tumor (ET) patients with residual disease. Bone Marrow Transplant 1997; 19:S86.
16. Ladenstein R, Lasset C, Pinkerton R, et al. Impact of megatherapy in children with high-risk Ewing's tumours in complete remission: a report from the EBMT Solid Tumour Registry. Bone Marrow Transplant 1995; 15:697-705.
17. Burdach S, Jürgens H, Peters C, et al. Myeloablative radiochemotherapy and hematopoietic stem-cell rescue in poor-prognosis Ewing's sarcoma. J Clin Oncol 1993; 11:1482-8.
18. Burdach S, Nurnberg W, Laws HJ, et al. Myeloablative therapy, stem cell rescue and gene transfer in advanced Ewing tumors. Bone Marrow Transplant 1996; 18 (Suppl. 1):S67-8.
19. Carli M, Colombatti R, Oberlin O, et al. High dose melphalan with autologous stem cell rescue in metastatic rhabdomyosarcoma. J Clin Oncol 1999; 17:2796-803.
20. Koscielniak E, Klingebiel TH, Peters C, et al. Do patients with metastatic and recurrent rhabdomyosarcoma benefit from high-dose therapy with hematopoietic rescue? Report of the German/Austrian Pediatric Bone Marrow Transplantation Group. Bone Marrow Transplantat 1997; 19:227-31.
21. Pollack IF. Brain tumours in children. N Engl J Med 1994; 331:1500-7.
22. Kalifa C, Hartmann O, Demeocq F, et al. High-dose busulfan and thiotepa with autologous bone marrow transpantation in childhood malignant brain tumors: a phase II study. Bone Marrow Transplant 1992; 9:227-33.
23. Graham ML, Herndon JE 2nd, Casey JR, et al. High dose chemotherapy with autologous stem cell rescue in patients with recurrent and high risk pediatric brain tumors. J Clin Oncol 1997; 15:1814-23.
24. Dunkel IJ, Boyett JM, Yates A, et al. High dose carboplatin, thiotepa and etoposide with autologous stem-cell rescue for patients with recurrent medulloblastoma. Children's Cancer Group. J Clin Oncol 1998; 16:222-8.
25. Dallorso S, Garrè ML, Cefalo G, et al. High dose therapy and peripheral blood progenitor cells rescue as treatment of poor risk central nervous system tumours. (Abstract). Paper presented at the International Symposium on Peripheral Blood Stem Cell Transplantations, September 5-8th 1999 Mulhouse, France.
26. Dallorso S, Dini G, Miano M, et al. G-CSF primed peripheral blood progenitor cells (PBPC) autotransplantation in stage IV neuroblastoma and poor risk solid tumors. Bone Marrow Transplant 1996; 18(S2): 182-4.
27. Eguchi H, Takawe Y, Kawano Y, et al. Peripheral blood stem cell autografts for the treatment of children over 1 year old with stage IV neuroblastoma: a long-term follow-up. Bone Marrow Trasplantat 1998; 21:1011-4.
28. Weaver CH, Hazelton B, Birch R, et al. An analysis of engraftment kinetics as a function of the CD 34 content of peripheral blood progenitor cell collections in 692 patients after the administration of myeloablative chemotherapy. Blood 1995; 86:3961-9.
29. Faucher C, Le Corroller AG, Chabannon C, et al. Autologous transplantation of blood stem cells mobilized with filgrastim alone in 93 patients with malignancies: the number of CD 34+ cells reinfused is the only factor predicting both granulocyte and platelet recovery. J Hematother 1996; 5:663-70.
30. Bensinger W, Appelbaum F, Rowley S, et al. Factors that influence collection and engraftment of autologous peripheral –blood stem cells. J Clin Oncol 1995; 13: 2547-55.
31. Ozkaynak MF, Matthay K, Cairo M, et al. Double-alkylator non-total-body irradiation regimen with autologous hematopoietic stem-cell transplantation in pediatric solid tumors. J Clin Oncol 1998; 16:937-44.
32. Grupp SA, Stern JW, Bunin N, et al. Tandem high-dose therapy in rapid sequence for children with high risk neuroblastoma. J Clin Oncol 2000; 18:2567-75.
33. Moss TJ, Cairo M, Santana VM, Weinthal J, Hurvitz C, Bostrom B. Clonogenicity of circulating neuroblastoma cells: implications regarding peripheral blood stem cell harvests. Br J Haematol 1996; 92:614-9.
34. Montanaro L, Pession A, Trerè D, et al. Detection of EWS chimeric transcripts by nested RT-PCR to allow reinfusion of uncontaminated peripheral blood stem cells in high risk Ewing's tumor in childhood. Haematologica 1999; 84:1012-5.

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Immunoablation followed or not by hematopoietic stem cells as an intense therapy for severe autoimmune diseases (SADS). New perspectives, new problems

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Estimates of the prevalence of autoimmune diseases (ADs) in Western countries range from 3%[1] to 6-7%[2] of the population. The list of ADs is increasing mainly because of better insight into the pathogenesis of several diseases long considered to be of unknown origin. Establishing the autoimmune basis of human disease may occasionally be arduous, but satisfactory criteria have been repeatedly proposed[3] and are generally utilized. Although autoimmunity has been thought of as the persistent failure of an integrated fabric of components rather than the consequence of specific forbidden clones,[4] in practice diseases may be confidently classified as autoimmune when they exhibit defined reactions against self-antigens as a major component of their pathogenesis. The intricacies of distinguishing between intrinsic and extrinsic etiologic and pathogenic mechanisms are compounded by the diversities inherent in each AD and even within the subsets of specific diseases.[5] It is not known whether the antibody response in systemic ADs is antigen-driven, such that the immune system is responding to self-proteins that have become autoantigenic,[6] or whether ADs represent a primary dysfunction of the immune system.[7] The two hypotheses are not mutually exclusive and the prevailing conception is that of a combination of genetic factors responding to environmental triggers,[8] these last including both exogenous and endogenous factors.

The majority of ADs are controlled, more or less satisfactorily, by conventional therapeutic manipulation of the immune system, but there is a hard core of refractory/relapsing, treatment-resistant[9] ADs for which the term "malignant autoimmunity" has appropriately been proposed.[10] As recently remarked by Mackay & Rose,[11] the holy grail of therapy is a targeted treatment that would specifically destroy the pathogenic clones responsible for ADs. That ideal remains unrealized.

Intense immunosuppression (*immunoablation*), followed by allogeneic or autologous hemolymphopoietic stem cell (HSC) transplantation, is a relatively new therapeutic approach, which was proposed for the first time by myself for the treatment of severe, refractory systemic lupus erythematosus (SLE).[12] Immunoablation has produced encouraging results in patients with ADs who have undergone allogeneic bone marrow transplantation because of coincidental hematologic malignancies. A great deal of prior research had already produced impressive results using transplant-based procedures in experimental animals (see later). Suggestions to carry these encouraging results into the clinic soon followed.[12,13] Reports of allogeneic transplants (allo-BMT) for coincidental diseases (ADs and malignancies) were published. Phase I and II clinical studies have followed through the efforts of the *European Group for Blood and Marrow Transplantation* (EBMT) and the *European League Against Rheumatism* (EULAR), and the *National Collaborative Study of Stem Cell Transplantation for Autoimmune Diseases*. A number of exhaustive reviews of the experimental[14-16] and clinical[8,17-24] aspects of these approaches have been published.

Results in animal models

This preclinical area is very extensive, and cannot be discussed in depth here. Following the first demonstration of transfer/cure of murine SLE in 1974,[25] the most important results of these experimental studies concern 1) the identity of the cellular elements responsible for the transfer of autoimmunity, 2) a possible graft-vs-autoimmunity effect following allo-BMT and 3) the therapeutic potential of autologous SCT. The first point is still controversial. It has been proposed that ADs, or at least experimental ADs, are *polyclonal stem cell diseases*.[15]

An important therapeutic effect of allo-BMT in leukemia and in other malignant diseases is the well-known graft-versus-leukemia effect.[26] A putative graft-versus-autoimmunity effect is supported by experiments showing that allogeneic

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chimerism achieved using a sublethal radiation conditioning regimen followed by allogeneic transplantation can prevent the onset of diabetes and even reverse pre-existing insulitis in non-obese diabetic (NOD) mice, whereas the same radiation protocol without allogeneic HSC is insufficient.[27] A similar effect has been shown using sublethal conditioning and an anti-CD154 monoclonal antibody.[28] These experimental findings support low-conditioning preparative regimens for allogeneic transplants also in ADs.[8,22,23]

An unexpected but provocative finding[14,16,21] was that autologous (and pseudoautologous) HSC transplantation is equally effective in curing murine adjuvant arthritis[29] and experimental autoimmune encephalomyelitis,[30] although allogeneic transplants proved superior in curing the latter disease.

Clinical results

Post-transplant autoimmunity

The term adoptive autoimmunity was proposed in 1992 to indicate the transfer of an autoimmune disorder from a HSC donor to a recipient.[31] If direct transmission of either pathogenetic lymphocytes or HSC that generate autoreactive clones from the donor can be demonstrated, the pathogenesis is clear. However, in many other instances, ADs can be attributed to the *immunological chaos*[32] or imbalance characterizing the post-transplant setting.

Resolution of pre-existing autoimmune disease following allogeneic bone marrow transplantation

In most such instances, patients with pre-existing ADs have developed a malignant disease of the blood requiring transplantation. If acquired aplastic anemia were classified as a *bona fide* autoimmune disease,[33] then of course it would represent the most common autoimmune disorder to be treated by allogeneic transplantation. However, this is a special condition that will be not discussed here.

Nine patients with rheumatoid arthritis (RA) received allo-BMT from HLA-identical sibling donors for severe aplastic anemia (SAA) occurring after gold salt therapy. They have been reviewed extensively elsewhere.[5,23,34-36] All patients entered remission, although 3 died of transplant-related mortality (TRM). Of the remaining 5 patients, 3 are in complete remission from their arthritis (one has been in complete remission for 20 years[35]), one developed a positive rheumatoid factor, and one relapsed 2 years after transplant even though the patient's immune system was 98.5% of donor origin.[37] Relapse was also observed in a patient with psoriasis and arthropathy following allogeneic transplantation.[38] The occurrence of relapse despite complete donor hemolymphopoietic reconstitution may be related to intrinsic susceptibility of the transplanted immune system (HLA-identical to the patient's) to powerful autoantigenic stimuli.

Between 1982 and 1992, 6 patients with Crohn's disease and leukemia underwent allogeneic marrow transplantation in Seattle.[39] One patient died of septicemia 97 days after transplant; the remaining 5 were observed for several years post-transplant (4.5, 5.8, 8.4, 9.9 and 15.3 years, respectively). Four of these 5 evaluable patients had no signs or symptoms of Crohn's disease post-transplant. Only one patient with mixed donor-host hematopoietic chimerism had a relapse of both Crohn's disease and chronic myeloid leukemia 1.5 years after transplantation.

Two patients with Evans' syndrome (ES), a combination of autoimmune hemolytic anemia (AIHA) and immune thrombocytopenic purpura, have received allogeneic transplants.[40] A 5-year old boy affected from infancy by relapsing, life-threatening ES was successfully transplanted with HLA-identical sibling cord blood.[41] There was total disappearance of autoantibodies, but the patient died after liver failure 9 months post-transplant. A child with thalassemia intermedia developed AIHA severe enough to promote an autologous transplant, had a short-lived remission, relapsed with a dramatic recurrence of hemolysis, and finally was cured following allo-BMT from an unrelated volunteer donor.[42] This might be the first clinical demonstration of the superior curing potential of allo- vs auto SCT.

Autologous transplants for the treatment of autoimmune disease

Autologous HSC transplants (ASCT), from marrow or now almost exclusively from peripheral blood, are much more commonly used to treat ADs than are allogeneic transplants for two reasons: the encouraging experimental results from Rotterdam[14,29,30] and from Jerusalem,[13,22] and the greater safety of the autologous procedures.[18,24,43,44] TRM at 2 years post-transplant for ADs was 8.6%, which is comparable to the procedure-related mortality following transplantation for non-Hodgkin's lymphoma (NHL).[45]

Contributing factors to higher than expected TRM may have been a learning curve for utilizing ASCT in new diseases, hitherto unrecognized hazards associated with profound immunodeficiency, especially following intense T-cell depletion, and unique organ dysfunction, such as heart and lung failure in systemic sclerosis.[46] A brief recapitulation of published reports follows.

Multiple sclerosis

MS is characterized by demyelination, immunophlogistic lesions around axons, and ultimately axon loss. Pathogenesis is widely held to be autoimmune,[47,48] with T-cell activity in the foreground.[49] It has become the most common disease treated by ASCT, mostly because of extensive pioneering work by Fassas *et al.*[50] Following an initial report, 24 patients with MS in progressive phase were conditioned with the BEAM regimen (carmustine, etoposide, cytosine arabinoside and melphalan). They then received autologous CD34+ progenitors that had been previously mobilized by cyclophosphamide (CY) and granulocyte-colony stimulating factor (G-CSF). They were also conditioned with antithymocyte globulin in order to deplete lymphocytes *in vivo*. One patient died of aspergillosis in the post-transplant period; the other 23 sustained no severe transplant-related morbidity. Improvement in disability, as measured with the Kurtzke Extended Disability Status Scale (EDSS), was seen in 10 patients and stabilization of MS occurred in 10 patients (43%). Following mobilization there was a significant decrease of gadolinium (Gd)-enhancing lesions on MRI imaging, and after ASCT out of 132 scans only 3 active lesions were found in 2 patients.[51] In another clinical study, 6 MS patients were treated with a conditioning regimen of CY (20 mg/kg) and total body irradiation (TBI) (12.6 Gy fractionated over 4 days). Peripheral blood CD34+ cells were mobilized with G-CSF. All patients experienced subjective and objective neurologic improvement.[52] There were no new Gd-enhancing lesions detected after transplantation. Another 11 patients were mobilized with CY (4 mg/m^2) and G-CSF, and 8 of them were autografted following the usual BEAM protocol.[54] There were significant improvements by the EDSS scale, and no fatalities. In addition to 2 autologous transplants, one patient with AML plus MS received an allogeneic transplant, with stabilization of MS at 48 months, and another had a syngeneic transplant, with stabilization of disease but no evidence of the oligoclonal bands in the CSF which were present before transplantation.[54]

In the GITMO-NEURO Intergroup ongoing study 10 cases of secondary progressive MS with EDSS initially between 5 and 6, a documented rapid progression over the last year unresponsive to conventional therapies and the presence of Gd-enhancing areas on brain MRI using a triple dose of Gd[55] underwent CD34+ mobilization and then ASCT following conditioning with BEAM.[56] Ten cases have undergone ASCT with a median follow–up of 9 months (range 2-30 months). No major serious adverse events were observed during and after treatment. Mobilization was successful in all cases, with a median number of 9.06x10^6/kg of CD34+ collected. During the 3-months pre-treatment period 346 Gd-enhancing areas/month/patient in the same period was 10.5 (range 1-38). The number of Gd-positive areas decreased dramatically already after mobilization with CY and dropped to 0 in 10 cases within one month from conditioning with BEAM (preliminary graph in ref. #23). All patients slightly improved clinically, or remained stable. The median EDSS decreased to 6 and the median Scripps' scale score increased to 70. In the first case MRI enhancing was still completely abrogated 30 months after transplantation. Although clinical amelioration and/or stabilization were observed, it was concluded that the final impact of this procedure on the natural history of the disease remains to be established in larger, possibly prospective randomized trials. Guidelines in a consensus report have been published.[57]

Rheumatoid arthritis

Following a dramatic amelioration in a single case,[58] 10 patients with RA have had autografts at St. Vincent's Hospital in Sidney, Australia, with no transplant-related mortality or serious toxicity.[59] Two cohorts of 4 patients each, with severe, active RA, received autologous unmanipulated HSC following conditioning with 100 and 200 mg/kg CY, respectively;[60] the subablative doses produced only transient responses, and superior results were obtained with the highest dose of CY. However in a prolonged study of 4 autologous transplant recipients with ADs (3 psoriasis, 1 RA) complicated by malignancies. ADs remitted in all of them but recurred at 8-24 months. It was suggested that a single autograft with non-T-cell-depleted HSC is unlikely to cure ADs.[60] In 4 patients with severe RA mobilization with CY (4 mg/m^2) was sufficient to confer significant improvement.[61] Other 4 patients were treated with CY 200 mg/m^2, ATG 90 mg/kg, and in 1 patient TBI 46 Gy, and autotransplanted with T-cell depleted CD34+ cells, but there was a relapse even in the irradiated patient.[62] A 39-year old patient is in CR following a syngeneic transplant, and his T-cell repertoire has become almost identical to that of the donor's.[63] Exhaustive reviews have been published.[34,36]

Juvenile chronic arthritis

Although the overall prognosis for children with juvenile chronic arthritis (JCA) is good, the disease is refractory and severely progressive in a small proportion of patients. Four such cases autotransplanted with marrow HSC have been reported,[64] but others have followed. The grafts

were purged with 2 cycles of TCD. The conditioning regimen include 4 days of ATG, CY 200 mg/kg and low grade (4 Gy) single dose fraction TBI. This intense conditioning regimen was well tolerated, and there was a substantial resolution of signs and symptoms of active disease, but there was also limited recurrence. One death was caused by post-transplant disseminated toxoplasmosis,[65] but others have also occurred. A so-called macrophage-activation syndrome (MAS) has been described in these patients, but there is no reason to distinguish it from the well-known hemophagocytic lymphohistiocytosis.[66,67]

Systemic lupus erythematosus

As originally suggested in 1993,[12] SLE is rapidly becoming another major target for autologous transplants. Four cases of concomitant SLE and malignancy have been published. They include chronic myeloid leukemia and SLE,[68] NHL and SLE,[69] and Hodgkin's disease and SLE.[70] In one case, the NHL did not relapse, but autoimmune thrombocytopenic purpura (AITP) supervened in association with an anticentromere antibody; the autoimmune disease thus appeared more refractory than the neoplasia.[71]

A number of concomitant SLE patients have undergone ASCT. Most of them have been reported in abstract form, and will not be discussed here. The first two cases were published in 1997.[72,73] As of this writing, there are 4 fully published cases of severe, relapsing/refractory SLE that have undergone intense immunosuppression followed by ASCT. The first case, with a 50-month follow-up, was transplanted with positively selected $CD34^+$ marrow cells after conditioning with thiotepa and CY, 50 mg/kg.[72] This patient is still in clinical remission 4 years after transplant, but there is a slow gradual reappearance of antinuclear antibodies (ANA), with a shift from a speckled to a homogeneous pattern. Also anti-ds DNA antibodies have appeared. In all the other cases PBSC were utilized following mobilization with CY-6-CSF; the CY dosage varied from 2 to 4 mg/m^2. In the Palermo case the patient had a refractory Evans' syndrome secondary to SLE that resolved after transplant.[74] The Paris case was conditioned with the BEAM regimen and had a continuous clinical remission, with a gradual reappearance of ANA.[75] In the most extensive clinical study published to date[76] 9 patients underwent stem-cell mobilization with CY 2 mg/m^2 and G-CSF10 mg/kg, 2 were excluded from transplantation because of infection (one death from disseminated mucormycosis), and 7 were autotransplanted after conditioning with CY (200 mg/kg), 1 g methylprednisolone, and 90 mg/kg equine antithymocyte globulin. All patients were seriously ill, with SLE diseases activity indices (SLEDAI) of 17-37, including 1 case with alveolar hemorrhage and 4 with WHO class III-IV glomerulonephritis and nephrotic syndrome. Lupus remained in clinical remission in all patients after transplant. ANA became negative, and spontaneous T-cell activation marker CD69 declined or normalized after transplantation.

Systemic sclerosis

Systemic sclerosis (SSc) of the diffuse type is a devastating disease in which pulmonary interstitial fibrosis in the most frequent cause of death.[77] Two transplants have been performed in Basel using CY 200 mg/kg and $CD34^+$ cell rescue, with moderate benefit.[78,79] Five patients in Seattle received treatment with CY 120 mg/kg, TBI 8 Gy and ATG 90 mg/kg followed by $CD34^+$ cell-selected autografts. The first 3 patients, followed for 13, 7 and 4 months showed no evidence of disease progression. Their skin scores, mobility, skin ulcers and arthalgias improved with a trend toward improvement in pulmonary function, although in one patient renal function deteriorated. One patient developed grade III non-infectious pulmonary toxicity.[80]

To date, the most successful case of autologous transplantation for SSc is that of a 13-year-old girl with severe, progressive lung involvement who underwent peripheral HSC transplantation after mobilization with CY and G-CSF, $CD34^+$ selection, conditioning with CY (200 mg/kg), and infusion of the monoclonal antibody Campath-G. Two years after transplantation, progressive and marked improvement had occurred; the pulmonary ground-glass opacities disappeared, the patient was steroid-independent, and there was an impressive improvement in growth rate.[81] In contrast, antinuclear and anti-Scl-70 antibody positivity remained substantially unchanged.

Evans' syndrome and autoimmune thrombocytopenic purpura

Refractory ES and refractory AITP that relapse after splenectomy and do not respond to corticosteroids are associated with substantial morbidity and mortality because of the combined effects of disease and treatment.[82] In a case report of a patient treated with ASCT, a 25-year old woman with ES received peripheral blood stem cell mobilization with routine doses of 4mg/m^2 CY and G-CSF; this was followed by exacerbation of hemolysis and thrombocytopenia, and the patient died of an intracranial hemorrhage.[83]

Four cases of refractory post-splenectomy relapsed AITP have been treated with intensive immunosuppression followed by ASCT. The first 2 cases responded dramatically[84] but then relapsed (*Lim, personal communication*). The other 2 cases did not respond at all.[85,86]

Special Issues

Conditioning

The main conditioning regimens are well known, and include CY 200 mg/kg over 4 days, the variant with thiotepa utilized in Genoa, and the equally well-known BEAM protocol, which has been found attractive for MS because of its intense lympholytic effect and the ability of BCNU and ARA-C metabolites to cross the (already disrupted) blood-brain barrier. Although the combination of chemotherapy with TBI has been shown to be significant risk factor for developing therapy-related AML/MDS,[87] van Bekkum is of the opinion that the combination with moderate-dose TBI is superior to chemotherapy alone.[88] AS already mentioned, this combination has been utilized for JCA.[64]

Intense immunosuppression without HSC rescue for treatment of autoimmune disease

Treatment with high-dose CY alone (200 mg/kg) has been used to treat severe aplastic anemia (SAA),[89] and has subsequently been extended to a spectrum of severe ADs[90] including Felty's syndrome (2 cases), AITP and ES (1 case each) and SLE. One patient with AITP experienced disease progression and died following high-dose CY. A patient with refractory demyelinating polyneuropathy that had been refractory to plasmapheresis had a complete remission. Hematologic reconstitution was similar to that generally found after autologous HSC rescue. This has been attributed to the fact that primitive HSC express high levels of aldehyde dehydrogenase, an enzyme responsible for cellular resistance to CY.[91]

Six patients with severe, relapsing SLE have also been treated with this regimen. Two are in complete, steroid-independent remission, one is in a partial remission, and three are showing *dramatic improvement* (although follow-up is currently less than 6 months). In one case of SLE,[92] the inadvertent administration of a single high dose of CY (5 g) resulted in a sustained remission, further confirming the efficacy of CY alone. However, the use of high-dose CY without potential back-up of cryopreserved SC could turn out to be hazardous in the context of multicenter trials.[36] The *ex vivo* expansion of progenitors could on the other hand significantly shorten the duration of neutropenia,[93] as has been impressively shown in patients autotransplantated for multiple myeloma.[94]

Use of T-cell depletion prior to HSC infusion in patients with autoimmune disease

Depletion of T-lymphocytes has been widely utilized in allotransplantation to reduce the incidence and severity of GVHD following allogeneic HSC transplants. Unfortunately, TCD is accompanied by many disadvantages, including a rise in graft rejection, leukemic relapse, and delayed immunologic reconstitution. New approaches that are being studied include the use of a higher proportion of donor HSC, selective T-cell subset depletion, and post-transplantation donor lymphocyte infusions (DLI). Because patients with active ADs are not in complete remission at the time of transplantation, van Bekkum[16,21] considers it mandatory to deplete the autograft of autoreactive lymphocytes. Most ADs are T-cell mediated and B-cell-mediated ADs[6,95] often display prominent T-cell dependency. Thus, TCD may be useful in the treatment of ADs. Theoretically, both activated and memory T- (and B-) lymphocytes should be eradicated, or at least maximally depleted. This can be achieved either by positive $CD34^+$ selection or by immunologic TCD. In addition TCD has been performed *in vivo* by administering ATG to the recipients. There is no indication of a potential threshold dose of T-cells acceptable for reinfusion. A 3-log depletion has been customary, but further depletion has been performed recently.[64,75] However marked TCD may be accompanied by late fungal and viral infections and lymphoproliferative disease. There seems little point in curing ADs at the cost of profound and permanent immunosuppression.[96]

Immune reconstitution following stem cell transplantation

Reconstitution of the immune system following either allogeneic or autologous transplantation has been studied extensively. Exhaustive reviews have been published.[97, 98]

The most common immunologic feature, also seen after intense chemotherapy, is a severe prolonged depression of CD^+ T-cells,[53,59,71,97-99] although in some cases $CD3^+$ T-cells have returned to pretransplantation levels after 10 months without disease relapse.[100] Age, prior TCD, radiation and other factors may all modulate thymic or extrathymic pathways and influence the rate and extent of T-cell recovery after transplantation. The sites of lymphoid reconstitution, whether thymic or extrathymic, in young and older patients has been the subject of abundant and frequently controversial literature.[97-101] The thymic output in adults following ASCT has been studied very recently utilizing the numbers of TCR-rearrangement excision circles (TREC) in peripheral blood T-cells 100%.[102] It was found that increases in concentrations of TREC post-transplant were associated with the development of broader CD4 T-cell TRC repertories, and that patients with no increases in TREC had limited and highly skewed repertories. The rela-

tive importance of thymus-dependent and thymus-independent pathways in adults is still controversial. The expanding CD4+ T-cell population may exhibit increased susceptibility to apoptosis.[103] It appears that also the infusion of large numbers of PBSC is not sufficient to produce T-cell immune competence, with special reference to the $CD4^+$ subpopulation.[104]

Discussion

Prevailing concepts of autoimmunity dictate that a stable cure of ADs can only be expected if the patients' autoreactive immunocompetent cells are replaced by healthy, non-autoreactive cells. The healthy immune cells must also remain unsusceptible to whatever phenomenon provoked the initial breakdown in tolerance.[22] Of the three approaches discussed here – allogeneic HSC transplantation, autologous HSC rescue following intense immunosuppression and intense immunosuppression alone- allogeneic HSC transplantation is theoretically the most promising. Allogeneic transplants have generally been followed by long-term remissions and possible cures. However mortality and morbidity associated with allogeneic transplantation, although decreasing steadily in other disease contexts,[105] is still unacceptable for most ADs. In addition there are reports of patients with RA relapsing despite complete or nearly complete donor immunologic reconstitution following allogeneic transplantation.[37,38] Leukemia relapse in donor cells is a rare but established occurrence following transplantation. Transfection and/or chromosomal fusion have been considered as possible explanations, but they seems quite improbable in the autoimmune setting, in which extrinsic events such as resensitization to autoantigens appear more probable. If relapses following allogeneic transplantation for ADs continue to be observed, the theoretical edge of an allogeneic procedure over an autologous transplant would be considerably weakened.[23] However the case report of a severe autoimmune hemolytic anemia having relapsed after ASCT but having achieved long-term clinical and immunologic CR following a MUD allo-BMT[81] is encouraging.

The recent introduction of minimally myelosuppressive regimens which avoid the devastating cytokine storm associated with the classical dose-intense conditioning regimens and exploit donor lymphocyte immune effects, is a promising development in the treatment of malignant and non-malignant diseases.[105-108] If a graft-versus-autoimmunity effect were to occur clinically, it might also prevent recapitulation of disease.[23,109] The simplest explanation for a similar effect consists in the progressive substitution of normal T- and B-cells in the place of autoreactive lymphocytes. However a selective elimination by cytotoxic lymphocytes of target autoimmune progenitor cells could also be envisaged, as has been elegantly shown in the case of $CD34^+$ CML progenitors.[110]

In the rare setting of an identical twin nonconcordant for disease a syngeneic transplant may be considered. A dramatic result following a syngeneic transplant in a patient with severe RA has been published[63] In the case of SLE only 23% of 66 monozygotic twins were found to be concordant for disease,[111] although a higher concordance has also been reported.[112] Concordance of antibody production is higher than disease concordance.[113] Also cord blood stem cells[43] may become an attractive option for the treatment of ADs.

Autologous transplantation has been hailed as a possible therapy for severe refractory ADs because of lower transplant-related mortality and greater feasibility.[5,18,24,40] In the EBMT registry, the overall survival at 2 years was 89±7%, with a median follow-up of 10 months for surviving patients. The transplant-related mortality at 2 years was 8±6%, which is comparable to that associated with ASCT for malignant disease.[45] Selection of patients with less severe disease could further reduce mortality, but on the other hand one must consider that the procedure is meant for refractory/relapsing patients who often have accumulated diffuse visceral damage.

Peripheral HSC are generally preferred to marrow HSC in almost all clinical situations, but very high doses of CY for mobilization should be discouraged. A dose of 4 mg/m^2 is generally utilized with adequate mobilization and minimal toxicity. These CY doses are immunosuppressive and may contribute to the efficacy of transplantation, as was clearly shown in MS[51,53,56] and in RA.[61]

A hitherto unsolved but fundamental question is whether intense immune suppression followed by ASCT is indeed capable of eradicating autoimmunity and thus inducing tolerance, or if the immune system remains fundamentally unaltered, and the so-called transplant is nothing more than a hematopoietic rescue. The first goal appears to have been achieved experimentally,[17,22] but in clinical settings what has been called *reprogramming the immune system*[112] has not been yet demonstrated. In SLE it has been proposed that the conditioning, with concurrent use of ATG, might provide *a window of time free of memory T-cell influence, during which the maturation of new lymphocyte progenitors may occur without recruitment to anti-self reactivity*.[76] In order to elucidate whether, if relapses occur, disease is reinitiated by lymphocytes surviving the conditioning regimen, or from the SC compartment, sophis-

ticated studies with gene-marked autologous SC are being performed.[115] If Shoenfeld's[116] concept of an idiotypic induction of autoimmunity is shown to be part of the etiology of SLE and other ADs, the impact of all these treatments would need further evaluation. Empirically, however, long-term remissions and relapses may also depend on the single disease and patient, but in most cases there is a distinct lowering of therapy-dependence, in addition to the resolution of severe/acute autoimmune *crises*. Whether this effect will prove to be superior to other immunosoppressive and/or immunomodulating treatments will have to be evaluated in prospective randomized trials, notwithstanding the problems inherent in recruiting sufficient numbers of homogenous patients. This may well be feasible for not infrequent diseases such as MS and RA, but will present many difficulties in other diseases such as SLE.

Even though the problem of the up to now excessive TRM will almost certainly be solved, the problem of late oncogenicity cannot be ignored, especially in younger patients with non-malignant diseases.[87] The risk of developing solid cancers was 3-4 times higher in patients treated with combined modality therapy during marrow transplantation than in controls.[117] In one study, a higher risk of acute myeloid leukemia was found following ASCT when the conditioning regimens included TBI.[118] In addition, some of these patients may have already been treated with prior chemotherapy, including large doses of alkylating agents, which has been shown to be the most important risk factor for developing MDS/AML. Preliminary cytogenetic screening could be useful to exclude patients already bearing chromosomal abnormalities.

Finally investigations using prospective randomized studies must be initiated. For example, in JCA, transplantation results should be compared with the prolonged CY pulse program that has been used recently in patients with severe JCA.[119]

In other diseases, such as MS, post-transplant treatment with ß-interferon could perhaps prolong transplant-induced remissions.[120] Even though ASCT has failed to produce clinical results comparable to the results achieved in animal models, some significant results have been achieved and future benefits are likely.

Conclusions

The excellent experimental results obtained with allogeneic and even autologous stem cell transplantation for ADs have given considerable impetus to similar treatments for refractory/ relapsing patients with severe ADs. Encouraging results following allogeneic SC transplantation have been reported in small numbers of patients with coexisting ADs and malignancies. However a few relapses have occurred despite donor immune cell engraftment. If a GVA effect is confirmed, the non-myeloablative allogeneic procedures could become extremely useful. In the meantime autologous transplantation using peripheral blood SC is currently being performed world-wide to treat ADs. Results are encouraging, but remissions rather than cures have been obtained. In some diseases, especially MS, results are superior to those obtained with conventional therapies. Long-term remissions have also been obtained by intense immunosuppression alone, demonstrating that autologous SC have mainly a rescue effect. Further clinical trials are clearly indicated.

References

1. Jacobson DL, Gange SJ, Rose NR, Graham NMH. Epidemiology and estimated population burden of slected autoimmune diseases In the United States. Clin Immunol Immunopathol 1997; 84:223-43.
2. Sinha AA, Lopez MT, Mc Devitt HO. Autoimmune diseases: the failure of self-tolerance. Science 1990; 248: 1380-5.
3. Rose NR. Foreword-the use of autoantibodies. In Autoantibodies, ed. JB Peter, Y Shoenfeld, 1996; Amsterdam: Elsevier pp.27-9.
4. Shoenfeld Y, Isenberg O. The mosaic of autoimmunity. Amsterdam: Elsevier 1989.
5. Marmont AM. Stem cell transplantation for severe autoimmune disorders, with special reference to rheumatic diseases. J Rheumatol 1997; 24 (Suppl. 48):13-8.
6. Tan EM. Autoantibodies and autoimmunity: a three decade perspective. In; B-Lymphocytes and Autoimmunity, Eds. N Chiorazzi, RG Lahita, K Pavelka, M Ferrarini. Ann NY Acad Sci 1997; 815:1-14.
7. Coutinho A. An outsider's view on SLE research. Lupus 1999; 8:171-3.
8. Nash RA. Prospects of stem cell transplantation in autoimmune diseases. J Clinical Immunol 2000; 20: 38-45.
9. Cash JM, Wilder RL, Eds. Treatment- Resistant Rheumatic Disease. Rheum Dis Clin N Am 1995; 21: 1-170.
10. Lafferty KL, Gazda LS. Costimulation and the regulation of autoimmunity, Eds. 1998. The Autoimmune Diseases. San Diego: Academic.
11. Mackay IR, Rose NR. Autoimmunity yesterday, today and tomorrow, Eds: The Autoimmune Diseases. San Diego: Academic, Press, 1998; pp. 849-72.
12. Marmont AM. Perspective: immunoablation with stem cell rescue: a possible cure for systemic lupus erythematosus. Lupus 1993; 2:151-6.
13. Slavin S. Treatment of life-threatening autoimmune disease with myeloablative doses of immunodepressive agents: experimental background and rationale for ABMT. Bone Marrow Transplant 1993; 12: 201-10.
14. Van Bekkum DW. Review: BMT in experimental autoimmune diseases. Bone Marrow Transplant 1993; 11:183-7.
15. Ikehara S. Bone marrow transplantation for autoimmune diseases. Acta Haematol 1998; 99:116-32.
16. Van Bekkum DW. Stem cell transplantation in exper-

imental models of autoimmune disease. J Clin Immunol 2000; 20:11-7.
17. Snowden JA, Brooks PM, Biggs JC. Haematopoietic stem cell transplantation for autoimmune diseases. Br J Haematol 1997; 99:9-22.
18. Marmont AM. Stem cell transplantation for severe autoimmune diseases : progress and problems. Haematologica 1998; 83:733-43.
19. Sherer Y, Schoenfeld Y. Stem cell transplantation – a cure for autoimmune diseases. Lupus 1998; 7:137-40.
20. Burt RK, Traynor A. Hematopoietic stem cell therapy of autoimmune diseases. Curr Opin Hematol 1998; 5: 472-7.
21. Van Bekkum DW. Short analytical review. New opportunities for treatment of severe autoimmune diseases: bone marrow transplantation. Clin Immunol Immunopathol 1998; 89:1-10.
22. Slavin S. Autologous and allogeneic stem cell transplantation for the treatment of autoimmune diseases as a potential new approach. In The Decade of Autoimmunity, Eds. Y Schoenfeld, 1999; pp. 399-408. Amsterdam: Elsevier.
23. Marmont AM. New horizons in the treatment of autoimmune diseases: immunoablation and stem cell transplantation. Ann Rev Med 2000; 51:115-34.
24. Burt R, Rowlings P, Traynor A. Hematopoietic Stem Cell Transplantation for Severe Autoimmune Disease: Know Thyself. In Ball E, Lister J, Law P (eds). Hematopoietic Stem Cell Therapy. 2000. Churchill Livingstone, New York: 203-15.
25. Morton JL. Transplantation of autoimmune potential. I. Development of antinuclear antibodies in H-2 histocompatible recipients of bone marrow from New Zealand Black mice. Proc Natl Acad Sci 1974; 71:2162-5.
26. Schlomchick WD, Emerson SG. The immunobiology of T cell therapies for leukemias. Acta Haematol 1996; 96:189-213.
27. Li H, Kaufman CL, Boggs SS, et al. Mixed allogeneic chimerism induced by a sublethal approach prevents autoimmune diabetes and reverses insulitis in nonobese diabetic (NOD) mice. J Immunol 1996; 156:380-7.
28. Jeung E, Iwakosh N, Woda BA, et al. Allogeneic hematopoietic chimerism in mice treated with sublethal myeloablation and anti-CD154 antibody: absence of graft-versus-host disease, induction of skin allograft tolerance, and prevention of recurrent autoimmunity in islet-allografted NOD/Lt mice. Blood 2000; 95:2175-82.
29. Knaan-Shanzer S, Houben P, Kinwel-Bohre EBM, van Bekkum DW. Remission induction of adjuvant arthritis in rats by total body irradiation and autologous bone marrow transplantation. Bone Marrow Transplant 1992; 8:333-8.
30. Van Gelder M, van Bekkum DW. Effective treatment of relapsing autoimmune encephalomyelitis with pseudoautologous bone marrow transplantation. Bone Marrow Transplant 1996; 18:1029-34.
31. Marmont AM. Autoimmunity and bone marrow transplantation. Bone Marrow Transplant 1992; 9:1-3.
32. Sherer Y, Shoenfeld Y. Autoimmune diseases and autoimmunity post-bone marrow transplantation. Bone Marrow Transplant 1998; 22:873-81.
33. Young NS. Hematopoietic cell destruction by immune mechanisms in acquired aplastic anemia. Semin Hematol 2000; 37:3-14.
34. Tyndall A, Milliken S. Bone marrow transplantation for rheumatoid arthritis. In: Established Rheumatoid Arthritis, ed. PL Van Riel, B Bresnihan. Baillère Best Pract Res Clin Rheumatol 1999; 13:719-35.
35. Nelson JL, Torrez R, Louie FM, et al. Pre-existing autoimmune disease in patients with long term survival after allogeneic bone marrow transplantation: Seattle experience. J Rheumatol 1997; 24 (Suppl. 48): 23-9.
36. Lowenthal RM, Graham SR. Does hemopoietic stem cell transplantation have a role in treatment of severe rheumatoid arthritis? J Clin Immunol 2000; 20:17-23.
37. McKendry RJR, Huebsch L, Le Clair B. Progression of rheumatoid arthritis following bone marrow transplantation: a case report with 13-years follow-up. Arthritis Rheum 1996; 39:1246-53.
38. Snowden JA, Kearney P, Keraney A, et al. Long-term outcome of autoimmune disease following allogeneic bone marrow transplantation. Arthritis Rheum 1998; 41:453-9.
39. Lopez Cubero SO, Sullivan KM, McDonald GB. Course of Crohn's disease after allogeneic bone marrow transplantation: report of 6 cases. Gastroenterology 1998; 114:433-40.
40. Marmont AM. Immune ablation and stem cell transplantation for severe Evans syndrome and refractory thrombocytopenic purpura. Bone Marrow Transplant 1999; 23:1215-6.
41. Raetz E, Beatty PG, Rose J. Treatment of severe Evans' syndrome with an allogeneic cord blood transplant. Bone Marrow Transplant 1997; 20:427-9.
42. Di Stefano P, Zecca M, Giorgiani G, et al. Resolution of immune hemolytic anaemia with allogeneic bone marrow transplantation after unsuccessful autograft. Br J Haematol 1999; 106:1063-4.
43. Tyndall A, Gratwohl A. Bood and marrow stem cell transplantation in autoimmune diseases: a consensus report written on behalf of European League against Rheumatism (EULAR) and the European Group for Blood and Marrow Transplantation. Bone Marrow Transplant 1997; 19:643-5.
44. Marmont AM, Tyndall A, Gratwohl A, Vischer A. Haemopoietic precursor- cell transplants for autoimmune diseases. Lancet 1995; 345:978.
45. Tyndall A, Fassas A, Passweg J, et al. Autologous hematopoietic stem cell transplantation for autoimmune disease-feasibility and transplant-related mortality. Bone Marrow Transplant 1999; 24:729-34.
46. Mc Sweeney PA, Furst DE, West SG. High-dose immunosuppressive therapy for rheumatoid arthritis: some answers, more questions. Arthritis Rheum 1999; 42:2269-74.
47. Steinman L. Multiple sclerosis: a coordinated immunological attack against myelin in the central nervous system. Cell 1996; 85:299-302.
48. Gulcher JR, Varranian T, Stefansson K. Is multiple sclerosis an autoimmune disease? Clin Neurosci 1994; 2:246-52.
49. Hohfeld R, Londi M, Massacesi L, Salvetti M. T-cell immunity in multiple sclerosis. Immunol Today 1995; 16:259-61.
50. Fassas A, Anagnostopoulos A, Kazis A, et al. Peripheral blood cell transplantation in the treatment of progressive multiple sclerosis: first results of a pilot study. Bone Marrow Transplant 1997; 20:631-8.
51. Fassas A, Anagnostopoulos A, Kazis A, et al. Autologous stem cell transplantation in progressive multiple sclerosis. An interim analysis of efficacy. J Clin Immunol 2000; 20:24-30.
52. Burt RK, Traynor A, Pope, et al. Treatment of autoimmune disease by intense immunosuppressive conditioning and autologous hematopoietic stem cell transplantation. Blood 1998; 92:3505-14.
53. Kozak T, Hardova E, Pit'ha J, et al. High-dose immunosuppressive therapy with PRBC support in the

treatment of poor risk systemic sclerosis. Bone Marrow Transplant 2000; 25:525-31.
54. Mandalfino P, Rice G, Smith A, et al. Bone marrow transplantation in multiple sclerosis. J Neurol 2000; 415:1-5.
55. Filippi M, Rovaris M, Capra R, et al. A multi-center longitudinal study comparing the sensitivity of monthly MRI after standard and triple dose gadolinium-DTPA for monitoring disease activity in multiple sclerosis. Brain 1998; 121:2011-20.
56. Mancardi GL, Saccardi R, Filippi M, Marmont AM for the Italian GITMO-Neuro Intergroup on Autologous Stem Cell Transplantation for Multiple Sclerosis. Abrogation of Gadolinium-enhanced MRI activity in severe cases of multiple sclerosis following autologous hematopoietic stem cell transplantation. Submitted.
57. Comi G, Kappos L, Clanet M, et al. Guidelines for autologous blood and marrow stem cell transplantation in multiple sclerosis: a consensus report written on behalf of the European Group for Blood and Marrow Transplantation and the European Charcot Foundation. J Neurol 2000; 247:376-82.
58. Joske DJ, Ma DTS, Langland DR, Owen ET. Autologous bone marrow transplantation for rheumatoid arthritis. Lancet 1997; 350:337-8.
59. Snowden JA, Biggs JC, Milliken ST, et al. A randomized, blinded, placebo-controlled, dose escalation study of the tolerability and efficacy of filgrastim for hematopoietic stem cell mobilisation in patients with severe active rheumatoid arthritis. Bone Marrow Transplant 1998; 22:1035-41.
60. Snowden JA, Biggs JC, Milliken ST, et al. A phase I-II escalation study of intensified cyclophosphamide and autologous blood stem cell rescue in severe, active rheumatoid arthritis. Arthritis Rheum 1999; 42:2286-92.
61. Breban M, Dougados M, Picard F, et al. Intensified dose (4 mg/mq) cyclophosphamide and granulocyte colony-stimulating factor administration for hematopoietic stem cell mobilisation in refractory rheumatoid arthritis. Arthritis Rheum 1999: 42:2275-80.
62. Burt RK, Georganas C, Schroeder J, et al. Autologous hematopoietic stem cell transplantation in refractory rheumatoid arthritis. Arthritis Rheum 1999; 42: 2281-5.
63. McColl G, Kashaka H, Wicks I. High-dose Chemotherapy and syngeneic hematopoietic stem-cell transplantation for severe, seronegative rheumatoid arthritis. Ann Intern Med 1999; 131:507-509.
64. Wulfraat N, van Royen A, Bierling M et al: Autologous haematopoietic stem cell transplantation in four patients with refractory juvenile chronic arthritis. Lancet 1999; 353:550-3.
65. Quartier P. Prieur AM, Fischer A. Haematopoietic stem cell transplantation for juvenile chronic arthritis. Lancet 1999; 353:1883-4.
66. Marmont AM, Spriano M. Hemophagocytic lymphohistiocytis: still a morphological diagnosis. Haematol ogy 1995; 80:480-1.
67. Janka G, Imashuku S, Elinder G, et al. Infection – and malignancy- associated hemophagocytic syndromes. Secondary hemophagocytic lymphohistiocytosis. Hematol Oncol Clin N Am 1998; 12:435-44.
68. Meloni G, Capria S, Vignetti M, Mandelli F. Blast crisis of chronic myelogenous leukemia in long-lasting systemic lupus erythematosus: regression of both diseases after autologous bone marrow transplantation. Blood 1997; 89:4650.
69. Snowden JA, Patton WN, O'Donnell JAL, et al. Prolonged remission of long-standing lupus erythematosus after autologous bone marrow transplant for non-Hodgink's lymphoma. Bone Marrow Transplant 1997; 19:1247-50.
70. Schachna L, Ryan PF, Schwarer AP. Malignancy-associated remission of systemic lupus erythematosus maintained by autologous peripheral blood stem cell transplantation. Arthritis Rheum 1998; 41:2271-2.
71. Euler HH, Marmont AM, Bacigalupo A, et al. Early recurrence or persistence of autoimmune disease after unmanipulated autologous stem cell transplantation. Blood 1996; 88:3621-5.
72. Marmont AM, van Lint MT, Gualandi F, Bacigalupo A. Autologous marrow stem cell transplantation for systemic lupus erythematosus of long duration. Lupus 1997; 2:151-6.
73. Burt RK, Traynor AE, Ramsey-Goldman R. Hematopoietic stem cell transplantation for systemic lupus erythematosus. N Engl J Med 1997; 357:1777-8.
74. Musso M, Porretto F, Crescimanno A, et al. Autologous peripheral blood stem and progenitor (CD 34+) cell transplantation for systemic lupus erythematosus complicated by Evans syndrome. Lupus 1998; 7:492-4.
75. Fouillard L, Gorin NC, Laporte JP, et al. Control of severe systemic lupus erythematosus after high-dose immunosuppressive therapy and transplantation of CD 34+ purified autologous stem cell from peripheral blood. Lupus 1999; 8:320-3.
76. Traynor AE, Schroeder J, Rosa RM, et al. Treatment of severe systemic lupus erythematosus with high dose chemotherapy and haemopoietic stem-cell transplantation: a phase I study. Lancet 2000; 356:701-7.
77. Medsyer TA, Steen VD. Classification, prognosis. In Systemic sclerosis, ed. PJ Clements, DE Furst, 1996; pp. 51-64. Baltimore, MD: Williams & Wilkins.
78. Tamm M, Gratwohl A, Tichelli A et al: Autologous haematopoietic stem cell transplantation in a patient with severe pulmonary hypertension complicating connective tissue disease. Ann Rheum Dis 1996; 55: 779-80.
79. Tyndall A, Black C, Finke J, et al. Treatment of systemic sclerosis with autologous haematopoietic cell transplantation. Lancet 1997; 349:254.
80. McSweeney PA; Furst DE, Storek J, et al. High-dose immunosuppressive therapy (HDIT) using total body irradiation (TBI), cyclophosphamide (CY) and ATG with autologous CD 34+ selected peripheral blood stem cell (PBSC) rescue as treatment for severe systemic sclerosis. Blood 1998; 92 (Suppl): 295 (Abstr.)
81. Martini A, Maccario R, Ravelli A, et al. Marked and sustained improvement two years after autologous stem cell transplantation in a girl with systemic sclerosis. Arthritis Rheum 1999; 42:807-11.
82. McMillan R. Therapy for adults with refractory chronic immune thrombocytopenic purpura. Ann Intern Med 1997; 126:307-14.
83. Martino R, Sureda A, Brunet S. Peripheral stem cell mobilization in a refractory autoimmune Evans syndrome: a cautionary case report. Bone Marrow Transplant 1997; 20:521.
84. Lim SH, Kell J, Al-Sabah A, et al. Peripheral blood stem-cell transplantation for refractory autoimmune thrombocytopenia. Lancet 1997; 40:475.
85. Skoda RC, Tichelli A, Tyndall A, et al. Autologous peripheral blood stem cell transplantation in a patient with chronic autoimmune thrombocytopenia. Br J Haematol 1997; 99:57.
86. Marmont AM, van Lint MT, Occhini D, et al. Failure of autologous stem cell transplantation in refractory thrombocytopenic purpura. Bone Marrow Transplant 1998; 22:827-8.
87. Pedersen-Bjergaard J, Andersen MK, Christiansen DH. Therapy-related acute myeloid leukemia and

myelodysplasia after high-dose chemotherapy and autologous stem cell transplantation. Blood 2000; 95: 3273-9.
88. Van Bekkum DW. Conditioning regimens for the treatment of experimental arthritis with autologous bone marrow transplantation. Bone Marrow Transplant 2000; 25:357-64.
89. Brodsky RA, Sensenbrenner LL, Jones RL. Complete remission in severe aplastic anemia after high dose cyclophosphamide without bone marrow transplantation. Blood 1996; 87:491-4.
90. Brodsky RA, Petri M, Douglas Smith B, et al. Immunoablative high dose cyclophosphamide without stem cell rescue for refractory, severe autoimmune disease. Ann Intern Med 1998; 129:1031-5.
91. Jones RJ, Collector MI, Barber JP et al: Characterization of mouse lymphohematopoietic stem cells lacking spleen colony-forming activity. Blood 1996; 88:487-91.
92. Mittal G, Balarishna C, Mangat G, et al. "Sustained remission" in a case of SLE following megadose cylophosphamide. Lupus 1998; 8:77-80.
93. Pecora AL. CD 34+ cell selection and ex vivo expansion in autologous and allogeneic transplantation. In Rowe JM, Lazarus HM, Carella AM (eds). Bone Marrow Transplantation 2000. Dunitz, London:1-19.
94. Reiffers J, Caillot C, Dazey B, et al. Abrogation of post-myeloablative chemotherapy neutropenia by ex vivo expanded autologous CD 34 positive cells. Lancet 1999; 354:1092-3.
95. Boitard C. B cells and autoantibody production in autoimmune diseases. Heidelberg: Springer-Landes 1996.
96. Krance R, Brenner M. BMT beats autoimmune disease. Nat Med 1998; 4:153-5.
97. Guillaume T, Rubinstein DB, Syman M. Immune reconstitution and immunotherapy after autologous hematopoietic stem cell transplantation. Blood 92:1471-90.
98. Mackall CL. T-cell immunodeficiency following cytotoxic antineoplastic therapy: a review. Stem Cells 2000; 18:10-18.
99. Durez P, Toungouz M, Schandence L. Remission and immune reconstitution after T-cell depleted stem cell transplantation for rheumatoid arthritis. Lancet 1998; 352:881.
100. Bomberger C, Singh Jairam M, Rodey G, et al. Lymphoid reconstitution after autologous PBSC transplantation with FACS-sorted CD 34 + hematopoietic progenitors. Blood 1998; 91:2588-600.
101. Heitger A, Neu N, Kern H, et al. Essential role of the thymus to reconstitute naive (CD4RA+) T-helper cells after human allogeneic bone marrow transplantation. Blood 1997; 90:850-7.
102. Douek DC, Vescio RA, Betts MR, et al. Assessment of thymic output in adults after haematopoietic stem cell transplantation and prediction of T-cell reconstitution. Lancet 2000; 355:1875-81.
103. Hakim FT, Capada R, Kaimei S, et al. Constraints on CD + recovery postchemotherapy in adults: thymicin-sufficiency and apoptotic decline of expanded peripheral CD + cells. Blood 1997; 90:3789-98.
104. Mackall CL, Stein D, Fleisher TA, et al. Prolonged CD4 depletion after sequential autologous peripheral blood progenitor cell infusions in children and young adults. Blood 2000; 96:754-62.
105. Frassoni F, Labopin M, Gluckman E, et al. Results of allogeneic bone marrow transplantation for acute leukaemia have improved over time in Europe. Bone Marrow Tranplant 1996; 17:13-8.
106. Slavin S, Nagler A, Naparstek E, et al. Nonmyeloablative stem cell transplantation and cell therapy as ana alternative to conventional bone marrow transplantation with lethal cytoreduction for the treatment of malignant and nonmalignant hematologic disease. Blood 1998; 91:756-63.
107. Khouri IF, Keating M, Korbling M, et al. Transplant-lite: induction of graft-versus-malignancy using fludarabine-based nonablative chemotherapy and allogeneic blood progenitor-cell transplantation as treatment for lymphoid malignancies. J Clin Oncol 1998; 16:2817-24.
108. Storb R. Nonmyeloablative preparative regimens: experimental data and clinical practice. Am Soc Clin Oncol Educ Book 1999; 241-9.
109. Marmont AM, van Bekkum DW. Stem cell transplantation for severe autoimmune diseases: new proposals but still unanswered questions. Bone Marrow Transplant 1995; 16:407-8.
110. Gao L, Bellantuono I, Elsasser E, et al. Selective elimination of leukemic CD34+ progenitor cells by cytotoxic T lymphoytes specific for WTI. Blood 2000; 95:2198-203.
111. Deapen DM, Escalante A, Weintraub L, et al. A revised estimate of twin concordance in systemic lupus erythematosus. Arthritis Rheum 1992; 35:311-8.
112. Arnett FC. The genetics of human lupus. In Dubois' Lupus Erythematosus, ed. DJ Wallace, BH Hahn, 1997; pp.77-117. Baltimore, MD: Williams & Wilkins.
113. Reichlin M. Systemic lupus erythematosus. In Rose NR, Mackay IR (eds): The Autoimmune Diseases, San Diego; Academic Press, 1998; pp. 283-98.
114. Waldmann H, Cobbold S: Reprogramming the immune system. In: autoimmune disease: aethopathogenesis, diagnosis and treatment, ed. BM Lydyard, J Brostoff, 1994; pp. 164-5. Oxford, UK: Blackwell.
115. Burt RK, Brenner M, Burns W et al. Gene-marked autologous hematopoietic stem cell transplantation of autoimmune disease. J Clin Immunol 2000; 20:1-9.
116. Shoenfeld Y. Eppur si muove (Galileo Galilei 1564-1642): the idiotypic dysregulation of autoantibodies as part of the etiology of SLE. Lupus 2000; 9: 481-3.
117. Curtis RE, Rowlings PA, Deeg HJ, et al. Solid cancers after bone marrow transplantation. N Engl J Med 1997; 336:897-904.
118. Dorrington DL, Vase JM, Anderson R, et al. Incidence and characterization of secondary mylodisplastic syndrome and acute myelogenous leukemia following high-dose radiochemotherapy and autologous stem cell transplantation for lymphoid malignancies. J Clin Oncol 12:2527-34.
119. Wallace C, Sherry DD. Trial of intravenous pulse cyclophosphamide and methilprenisolone in the treatment of severe systemic-onset juvenile rheumatoid arthritis. Arthritis Rheum 1997; 40:1852-5.
120. PRISM Study Group. Randomised double-blind placebo-controlled study of interferon beta-1a in relapsing/remitting multiple sclerosis. Lancet 1998; 52: 1491-7.

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Immunoablation followed by autologous hematopoietic stem cell infusion for the treatment of severe autoimmune disease

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ABSTRACT

Background and Objectives. **The aim of this study was to evaluate the tolerability and effectiveness of a non-myeloablative conditioning regimen followed by autologous hematopoietic stem cell infusion for the treatment of severe autoimmune diseases.**

Design and Methods**. From 1996 patients with severe autoimmune disease not responsive to conventional immunosuppressive treatment were selected. The patients' blood or marrow cells were harvested after incubation with vincristine and methylprednisolone. Two different immunoablative conditioning regimens were employed. The first used cyclophosphamide (2500 mg/m² in one day) and antilymphocyte globulin (ALG) (15 vials/m² in three days) and the second used fludarabine (300 mg/m² in two courses of 5 days) plus ALG (25 vials/m² in 5 days).**

Results. **Nineteen patients (14 female, 5 male) with severe autoimmune diseases were treated. Nine had a rheumatologic disorder (5 juvenile chronic arthritis, 1 rheumatoid arthritis, 1 systemic vasculitis, 1 Sjögren's syndrome, 1 Behçet's disease), 4 a neurologic disorder (3 multiple sclerosis, 1 myasthenia), 3 a haematologic disease (2 pure red cell aplasia, 1 autoimmune thrombocytopenia), 2 had a gastrointestinal disease (1 Crohn's disease, 1 autoimmune enteropathy) and 1 had a multiple autoimmune disorder. There was no regimen-related toxicity and no opportunistic infections occurred. Ninety percent of the patients improved and/or had a complete remission after the procedure. Fifty percent of the subjects went into complete or partial remission after a median follow-up of 15 months (range 3-25) while 50% relapsed after a median follow-up of 11 months, (range 6-16). The incidence of relapse in the group treated with fludarabine was lower (30%).**

Interpretation and Conclusions**. A non-myeloablative conditioning regimen was able to induce persistent remission in some patients with severe autoimmune diseases. There was no mortality or morbidity related to the procedure. The extent of remission does, however, remain to be established.**

Key words: immunoablation, fludarabine, autologous bone marrow transplantation, autoimmune diseases

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Despite the results obtained in recent years with immunosuppressive therapy, some systemic autoimmune diseases still have considerable morbidity and mortality with a poor quality of life and a severe prognosis.[1] With regards to rheumatoid arthritis, the most prevalent of these conditions, 50% of patients are still unable to work 10 years after the onset of the disease and, on average, their life-expectancy is reduced by 5-10 years.[2]

In animal models both allogeneic and autologous bone marrow transplants were able to cure some autoimmune diseases.[3-5] In human-beings there are reports about allogeneic and autologous stem cell transplantation, performed for a coexisting hematologic condition (leukemia, lymphoma or drug-induced bone marrow aplasia) which cured patients with rheumatoid arthritis and other autoimmune disorders or resulted in a long-lasting remission.[6,7]

The rationale for using hematopoietic stem cell transplantation (HSCT) to treat autoimmune diseases is based on the concept of ablation of an aberrant immune system followed by a reconstitution of a new immune system deriving from either an allogeneic donor or a T-cell depleted autologous transplant.[8]

The procedure-related mortality of allogeneic bone marrow transplantation (BMT) of over 10% cannot justify this particular approach whereas the comparative risk is less than 5% in an autologous setting.[9] A consensus report by the *European League against Rheumatism* (EULAR) and the *European Group of Blood and Marrow Transplantation* (EBMT) regarding marrow and stem cell transplantation in autoimmune diseases has been published and an international database has been set up.[10] By August 2000 the registry had collected data on more than 300 patients from all over the world (Tyndall, personal communication). Studies on small series of patients affected by multiple sclerosis, systemic lupus erythematosus, rheumatoid arthritis, systemic sclerosis and juvenile chronic arthritis have gradually been appearing in the literature: the results are promising results although the follow-ups are short.[11-15]

In this paper we investigated the tolerability and effectiveness of a non-myeloablative conditioning regimen followed by infusion of treated autologous hematopoietic stem cells in a cohort of 19 patients affected by a severe autoimmune disease.

Design and Methods

Patient selection

Juvenile chronic arthritis (JCA). Patients had a clinical diagnosis according to criteria established by the *American College of Rheumatology*,[16] a disease refractory to non-steroidal anti-inflammatory drugs and immunosuppressive drugs such as methotrexate, cyclosporine, cyclophosphamide and prednisone and adverse effects from long-term treatment with second-line anti-rheumatic drugs.

Rheumatoid arthritis (RA). Patients had to fulfil all the following criteria: a diagnosis of RA established according to the criteria of the *American College of Rheumatology*, a positive rheumatoid factor and failure of at least two disease-modifying agents (methotrexate, gold, penicillamine and hydroxychloroquine)

Systemic vasculitis. Patients with systemic leukocytoclastic vasculitis with cutaneous, intestinal and cerebral involvement non-responsive to conventional treatment including plasmapheresis.

Behçet's disease. Patients with muco-cutaneous disease and polyarthritis not responsive to steroids, colchicine, cyclosporine and thalidomide with a relapsing course.

Sjögren's syndrome. Patients with a clinical diagnosis confirmed by biopsy and positive to antinuclear antibody (ANA) and rheumatoid factor (RF) and severe pulmonary involvement not responsive to corticosteroids.

Multiple sclerosis (MS). Patients had clinically defined MS according to Poser's criteria[17] with progressive disease not stabilized by the administration of intravenous methylprednisolone and a Kurtzke extended disability status scale (EDSS)[18] score of 5.0 to 8.0 with evidence of at least 1.0 point deterioration in the last 12 months.

Myasthenia gravis. Eligible patients had progressive disease not responsive to conventional treatment (steroids, plasmapheresis) and involvement of respiratory muscles.

Acquired pure red cell anemia (PRCA). Patients fulfilled the following criteria: an autoimmune pathogenesis confirmed by the presence of erythropoietin-inhibiting antibody, transfusion-dependent disease refractory to steroids, cyclophosphamide and azathioprine.

Chronic autoimmune thrombocytopenia (AITP). Patients with life-threatening thrombocytopenia not responsive to conventional treatment (steroids, cyclophosphamide, high dose immunoglobulins, splenectomy).

Autoimmune enteropathy. Patients with chronic intractable diarrhea with anti-enterocyte antibodies not responsive to various immunosuppressive treatments (steroids, cyclosporine, azathioprine) and dependent on total parenteral nutrition.

Crohn's disease. Patients with total gastrointestinal disease, corticosteroid-dependent and not responsive to immunosuppressive treatment (azathioprine, cyclosporine, thalidomide, etanercept) with severe adverse effects from long-term steroid treatment.

Multiple expression autoimmune disease (*MEAD*). Patients with a association of desquamative erythroderma, insulin-dependent diabetes mellitus, autoimmune hemolytic anemia, interstitial pneumopathy, psoriasis and alopecia; the disease was corticosteroid-dependent with severe adverse effects from long-term steroid treatment.

Blood and bone marrow stem cell collection and treatment

Bone marrow stem cells were collected by conventional aspiration without priming. Peripheral blood stem cells were harvested after priming with granulocyte colony-stimulating factor (G-CSF) 10 mg/kg for only three days. In all patients the cells were treated with vincristine and methylprednisolone. *In vitro* this treatment inhibits Th1 and Th2-like responses, inhibits mixed lymphocyte culture response (MLC) and reduces the proportion of antigen-specific cytotoxic T-lymphocytes (CTL) without any increase in apoptosis.[19,20] In allogeneic 2-3 mismatched transplants with this treatment there was a reduction in the incidence of severe graft-versus-host disease (GVHD). Thereafter the suspension was mixed with the same volume of 20% DMSO solution in saline and cryopreserved plasma and stored in liquid nitrogen.

Conditioning regimen

The first group of 8 patients received 2500 mg/m^2 of cyclophosphamide on day –4 and 5 vials/m^2/day of lymphoglobulin (Inst. Merrieux) on day –3, -2,-1. Patients with multiple sclerosis also received cytosine arabinoside (1,750 mg/m^2 for 1 day) and dexamethasone (15 mg/m^2/day for 4 days). The second group of 11 patients was treated with a first course of fludarabine 30 mg/m^2/day for 5 days followed, one month later, by another course of fludarabine at the same dosage and 5 vials of ALG/m^2/day for 5 days. Patients with multiple sclerosis also received dexamethasone (15 mg/m^2/day for 4 days). The prophylaxis against chemotherapy toxicity entailed hyperhydration (3,000 mL fluids/m^2/day) and uromitexane by continuous infusion. The adverse reactions to horse serum were treated prophylactically with cortisone and anti-histamines. The

Table 1. Rheumatologic disorders.

Disease	Age/ sex (yrs)	Disease duration (yrs)	Conditioning regimen	Follow-up 3 mos	Follow-up 6 mos	Last Follow-up/mos
JCA	9 F	3	Cy/ALG	CR	CR	REL / 8
JCA	15 F	12	Cy/ALG	PR	REL	
JCA	15 F	6	Cy/ALG	CR	REL	
JCA	20 M	5	FLU/ALG	CR	CR	REL / 18
JCA	14 F	3	FLU/ALG	CR	CR	REL / 12
VASCULITIS	17 F	11	Cy/ALG	PR	PR	REL/ 13
RA	45 F	19	FLU/ALG	CR	CR	REL/ 12
SJÖGREN	46 F	4	FLU/ALG	CR	CR	CR/ 12
BEHÇET'S	33 F	3	FLU/ALG	CR	CR	CR/ 15

CR: complete remission; PR: response >50%; REL: relapse.

cells were infused 4 hours after ALG therapy had been completed.

All patients received cyclosporine (3 mg/kg/day) intravenous from day –1 until the seventh day when it was given orally.

Approval for the procedure was given by the institutional Ethics Committee and written consent was obtained from the patients or their families.

Results

To date 19 patients (5 M, 14 F) have been treated for severe autoimmune diseases. Their median age was 23.4 years (range 3-47), 10 were children. The source of cells was peripheral blood in 13 cases and marrow in 6. Nine patients were suffering from a rheumatologic disorder (5 JCA, 1 RA, 1 vasculitis, 1 Behçet's disease, 1 Sjögren's syndrome), 4 suffered from a neurologic disorder (3 MS, 1 myasthenia gravis), 3 from a hematologic disease (2 PRCA, 1 AITP), 2 from a gastrointestinal disorder (1 IBD, 1 autoimmune enteropathy) and 1 multiple expression autoimmune disease.

Toxicity

Non-hematologic toxicity was limited to grade 1 severity in the gastrointestinal system (nausea, vomiting) according to WHO toxicity criteria. One patient with MS presented a transient elevation of bilirubin (grade 3); 7 patients experienced neutropenia (neutrophil count < $0.5x10^9$/L) lasting a few days (median 3 days, range 2-5); during the period of neutropenia 1 patient had a positive blood culture for *Staphylococcus epidermidis*. The patient with systemic vasculitis had a flare up of symptoms during ALG infusion and required three blood-derivative transfusions.

After the procedure no post-transplant opportunistic infections occurred. The median time of hospitalization, including conditioning, was 12 days (range 8-20).

Clinical outcome

JCA. One patient had a systemic form while the other 4 had a systemic and polyarticular form. All had severe disease resistant to any treatment. The disease evolution was followed by the factors described by Giannini, including joint-swelling scores, pain scores and erithrocyte sedimentation rate (ESR).[21] All drug administration was stopped before the transplant and prednisone was tapered down and stopped within two months after HSCT. Four patients achieved complete remission and one a partial remission lasting at least 6 months. All patients relapsed between 6 and 18 months after HSCT (Table 1).

Systemic vasculitis. The patient had a severe life-threatening systemic leukocytoclastic vasculitis for 11 years with cutaneous, intestinal and cerebral involvement. The disease evolution was followed clinically and with serial measurements of ESR and fibrin degradation products. All drugs were stopped before the procedure. After the procedure prednisone was tapered down and stopped within two months; in addition monthly plasmaphereses were performed. The girl achieved a partial remission for 13 months then relapsed (see Table 1).

RA. This patient was a 45-year old woman diagnosed as having RA 19 years before the procedure. Assessment parameters were joint-swelling scores, patient's assessment of pain and acute-phase reactant values.[22] After HSCT prednisone was tapered down and stopped within 2 months. Complete remission was achieved and maintained for 12 months before the patient relapsed (see Table 1).

Sjögren's syndrome. The patient had severe pulmonary involvement with a low forced vital capacity (FVC) (2.2 L); after the procedure FVC increased markedly (3.2 L) and, for the first time since disease onset, ANA were normal and RF absent. Twelve months after the HSCT the patient is still in complete remission, being treated only with cyclosporine (Table 1).

Behçet's disease. The patient presented all the major diagnosis criteria and a severe polyarthritis. After the procedure she had a complete remission of all symptoms that continued at the last follow-up 15 months after the procedure (Table 1).

MS. All 3 patients had rapidly progressive disease despite immunosuppressive therapy during the year before transplant. After HSCT two of them had subjective and objective neurologic improvement defined as a decrease in the Kurtzke EDSS by at least 1 point after a follow-up of 3 and 24 months. The third patient showed no response and died 3 months after the procedure due to disease progression (see Table 2).

Myasthenia gravis. This patient was completely paralyzed and, because the involvement of respiratory muscles, underwent a tracheostomy. One month after HSCT the tracheostomy was closed. By the end of the second month she was

Table 2. Neurologic disorders.

Disease	Age/ sex (yrs)	Disease duration (yrs)	Conditioning regimen	Follow-up 3 mos	Follow-up 6 mos	Last Follow-up/mos
MS	22 M	1	Cy/Ara-C/DXM/ALG	PR	PR	PR/24
MS	46 F	23	FLU/DXM/ALG	NR		
MS	40 M	10	FLU/DXM/ALG	PR		
MYASTHENIA	47 F	28	Cy/ALG	PR	PR	PR/12

CR: complete remission; PR: response >50%; REL: relapse; NR: no response.

Table 3. Hematologic disorders.

Disease	Age/ sex (yrs)	Disease duration (yrs)	Conditioning regimen	Follow-up 3 mos	Follow-up 6 mos	Last follow-up (mos)
PRCA	44 M	3	Cy/ALG	CR	CR	REL/ 8
PRCA	7 F	2	FLU/ALG	NR		
AITP	4 F	3	FLU/ALG	CR	CR	CR/ 25

CR: complete remission; PR: response >50%; REL: relapse; NR: no response.

able to move normally and had a markedly improved electromyogram. She is still in partial remission after 12 months (Table 2).

PRCA. Two patients with very severe disease not responsive to steroids, cyclophosphamide, ALG and cyclosporine, requiring blood transfusions every 15-20 days. The first patient became transfusion-free for 6 months with an increased reticulocyte count but relapsed after 6 months. The second patient showed no response to the procedure (see Table 3).

AITP. A patient with a life-threatening condition with a fixed platelet level of $<10x10^9$/L refractory to current therapies including splenectomy performed after a cerebral hemorrhage. One month after the procedure the platelet level was $>100x10^9$/L. Prednisone was gradually tapered down and stopped after 5 months. At the last follow-up, 25 months after HSCT, the patient had a platelet count of $250x10^9$/L having maintained a level above $200x10^9$/L at the times (Table 3).

IBD. The patient had been affected by severe Crohn's disease, involving the entire gastrointestinal tract, since the age of one year. The disease was steroid-dependent and not responsive to azathioprine, cyclosporine, thalidomide, enteral nutrition, or etanercept. The patient had severe side-effects due to long-term steroid treatment. Six months after HSCT the disease was limited to the large bowel and required small doses of steroids (see Table 4).

Autoimmune enteropathy. This patient had received total parenteral nutrition since her first years of life. After the procedure her parameters of intestinal absorption improved (ratio mannitol/lactulose, xylose test) without any modification of intestinal biopsy or any clinical advantage.

Table 4. Gastrointestinal disorders.

Disease	Age/ sex (yrs)	Disease duration (yrs)	Conditioning regimen	Follow-up 3 mos	Follow-up 6 mos	Last Follow-up/mos
Auto-Entero.	14 F	13	Cy/ALG	PR	PR	REL/ 12
Crohn's disease	3 F	2	FLU/ALG	PR	PR	

Auto-Entero: Autoimmune enteropathy; CR: complete remission; PR: response >50%; REL: relapse.

MEAD. After the procedure the patient's respiratory function improved (increased Sa02 without needing oxygen supplementation). The hemolytic autoimmune anemia disappeared and the patient had a negative Coombs' test. Cortisone was gradually tapered down with resumption of growth but, after 22 months, it was not possible to interrupt all therapy. No improvement in the IDDM was noted.

Discussion

The first objective of this study was to assess the feasibility and toxicity of a non-myeloablative conditioning regimen followed by infusion of autologous *ex vivo* treated hematopoietic stem cells in a cohort of patients, mostly in pediatric age, with severe autoimmune diseases. The procedure was well tolerated; the patients did not suffer any major complications either during the conditioning or after the transplant and spent a short timein hospital. There was no treatment-related mortality; this compares with the 8% of TRM of the EBMT/EULAR database and the unacceptable 20% in a subgroup of children affected by juvenile chronic arthritis.[23,24] In terms of effectiveness all but two patients achieved a partial or complete remission but the high rate of early relapse (after a median of 12 months) in the first group treated with low-dose cyclophosphamide led to the cytoxan being replaced by a more immunosuppressive fludarabine-based conditioning regimen. Fludarabine, a nucleotide analog which targets both resting and proliferating lymphocytes, proved to be very effective in the treatment of autoimmune diseases.[25] In the second group of eleven patients 60% are in complete or partial remission after a median follow-up of 14 months (range 3-25 months).

In comparison to other published experiences the number of relapses seems higher but counterbalanced by a lower incidence of undesirable effects. For pediatric patients, who would otherwise suffer persistent handicap by continuous progression of disease, even a short-lived remission can be considered an advantage, especial-

ly in terms of steroid-sparing and the opportunity of restarting growth. Moreover this procedure was well tolerated by patients and could be carried out repeatedly in the same subject.

Since autologous transplantation cannot modify the genetic status of a patient and T- and B-lymphocytes play a central role in the pathogenesis of autoimmune diseases, the target of the procedure was only lymphoid tissue and both ablative chemotherapy and pharmacologic modulation were aimed at this issue. For this reason the type of blood cell that needed to be infused was the lymphocyte, and the number of $CD34^+$ cells infused was almost irrelevant. The hypothesis that *ex vivo* treament with vincristine and methylprednisolone induces functional paralysis of lymphocytes followed by an immune tolerance, proven in the recipient of mismatched bone marrow,[27] can only be suggested in the autologous setting. At this point we cannot rule out the possibility that re-infusion of autologous cells could be irrelevant to the outcome and the remission is, in fact, secondary only to intense immunosuppressive treatment.

Only randomized trials can confirm the hypothesis that autologous stem cell transplantation offers some advantage over intensive immunoablation without re-infusion of stem cells. Such trials should determine the conditioning regimen with lowest toxicity and identify which subset of patients with autoimmune disease may require an allogeneic graft from a sibling in order to be cured.

References

1. Cash JM, Wilder RM. Treatment-resistant rheumatic disease. Rheum Dis Clin N Am 1995; 21:1-270.
2. Markenson JA. World wide trends in the socioeconomic impact and long term prognosis of rheumatoid arthritis. Sem Arthritis Rheu 1991; 21:4-12.
3. Van Bekkum DW, Kinwell-Bohre EPM, Houben PFJ et al. Regression of adjuvant arthritis in rats following bone-marrow transplantation. Proc Soc Natl Sci 1989; 86:1090-4.
4. Knaan-Shanzer S, Houben P, Kinwel-Bohre, et al. Remission induction of adjuvant arthritis in rats by total body irradiation and autologous bone marrow transplantation. Bone Marrow Transplant 1992; 9:31-4.
5. Van Bekkum DW. BMT in experimental autoimmune diseases. Bone Marrow Transplant 1993; 11:183-7.
6. Liu yn JA, Jowitt SN. Resolution of immune mediate diseases following allogeneic bone marrow transplantation for leukemia. Bone Marrow Transplant 1992; 9:31-4.
7. Lowenthal RM, Cohen ML, Atkinson K, Biggs JC. Apparent cure of rheumatoid arthritis by bone marrow transplantation. J Rheumatol 1993; 20:137-40.
8. Marmont A. Immunoablation followed by allogeneic or autologous bone marrow transplantation. A new treatment for severe autoimmune diseases? Stem Cell 1994; 12:125-35.
9. Atkinson K, Dodds A, Miliken S, et al. Autologous blood stem cell transplantation: treatment related mortality of 2%. Aust NZ J Med 1995; 98:589-91.
10. Tyndall A, Gratwohl A, Blood and marrow stem cell transplant in autoimmune disease: a consensus report written on behalf of EULAR and EBMT. Bone Marrow Transplant 1997; 19:643-5.
11. Fassas A, Anagnostopoulos A, Kazis A. Peripheral blood stem cell transplantation in the treatment of progressive multiple sclerosis: first results of a pilot study. Bone Marrow Transplant 1997; 20:631-8.
12. Burt R, Traynor A, Pope R, et al. Treatment of autoimmune disease by intense immunosuppressive conditioning and autologous hematopoietic stem cell transplantation. Blood 1998; 92:3505-514.
13. Martini A, Maccario R, Ravelli A. Marked and sustained improvement after autologous stem cell transplant in a girl with systemic sclerosis. Arthritis Rheum 1999; 42:807-11.
14. Traynor A, Schroeder J, Rosa R, et al. Treatment of severe SLE with high-dose chemotherapy and hematopoietic stem-cell transplantation: a phase I study. Lancet 2000; 356:701-7.
15. Wulfraat N, van Royen A, Bierings M, et al. Autologous hematopoietic stem-cell transplantation in four patients with refractory juvenile chronic arthritis. Lancet 1999; 353:550-3.
16. Jacobsson LT, Knowler WC, Pillemer S, et al. American college of rheumatology 1987 criteria for rheumatoid arthritis. Arthritis Rheum. 1994; 37:1479.
17. Poser CM, Paty DW, Scheinberg L, et al. New diagnostic criteria for multiple sclerosis: guidelines for research protocols. Ann Neurol 1983; 13:227-31.
18. Kurtzke JF. Rating neurological impairment in multiple sclerosis: an expanded disability status scale (EDSS). Neurology 1983; 33:1444-52.
19. Lanfranchi A, Andolina M, Tettoni K, et al. Functional depletion of T cells by vincristine and methylprednisolone as in vitro model for the prevention of graft versus host disease. Haematologica 1992; 77:11-5.
20. Fragonas A, Perticarari S, Presani G, et al. In vitro Th1/Th2 modulation with vincristine and methylprednisolone: a possibly useful procedure for prevention of graft versus-host disease. Haematologica 2000, in press.
21. Giannini EH, Ruperto N, Ravelli A, et al. Preliminary definition of improvement in juvenile arthritis. Arthritis Rheum 1997; 40:1202-9.
22. Felson DT, Anderson JJ, Boers M, et al. American college of Rheumatology preliminary definition of improvement in rheumatoid arthritis. Arthritis Rheum 1995; 38:727.
23. Tyndall A, Fassas A, Passweg J, et al. Autologous haematopietic stem cell transplant for autoimmune diseases; feasibility and transplant-related mortality. Bone Marrow Transplant 1999; 24:729-34.
24. Vlieger AM; Brinkman D, Quartier P. Infection associated MAS in 3 patients receiving ASCT for juvenile chronic arthritis. Bone Marrow Transplant 2000; 25: S80.
25. Davis J, Fessler B, Tassiulas J, et al. High doses versus Low dose Fludarabine in the treatment of patients with severe refractory rheumatoid arthritis. J Rheumatol 1998; 25: 1694-704.
26. Brodsky RA, Petri M, Smith BD, et al. Immunoablative high doses cyclophosphamide without stem-cell rescue for refractory severe autoimmune diseases. Ann Intern Med 1998; 129:1031-5.
27. De Manzini A, Andolina M, Agosti E, et al. Low incidence of GvHD and rejection after pharmacological ex vivo modulation of bone marrow in 2-3 antigen mismatched BMT. Bone Marrow Transplant 1993; 11:114-7.

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In vitro incubation of bone marrow and peripheral stem cells with vincristine and methylprednisolone: functional T-cell depletion for haploidentical and autologous transplants

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ABSTRACT

A mismatched bone marrow transplantation is feasible only if the donor's marrow lymphocytes are eliminated from the graft. This can be achieved by several methods, but all have the disadvantage of inducing a long-lasting immune deficiency while the risk of graft rejection and leukemic relapse increase. We use a sort of functional T-cell depletion by treating the cells with vincristine and methylprednisolone. This method is surely the cheapest and has allowed us to perform 60 transplants with a tolerable risk of GVHD. The treatment of the donor's lymphocytes has already been demonstrated to be able to block the mixed lymphocyte culture reaction *in vitro*. In this experiment Th1 and Th2 activities were almost completely blocked without reduction of lymphocyte viability and apoptosis induction.

Key words: ABMT, treatment

Graft-versus-host-disease (GVHD) is a major cause of mortality and morbidity after allogeneic bone marrow transplantation particularly in mismatched transplants, but can be avoided by removing T-lymphocytes from the donor bone marrow.[1] However T-cell depletion increases the risk of graft rejection, as well as the chances of leukemic relapse.[2,3] In a previous study the effect of *in vitro* treatment with vincristine (VCR) and methylprednisolone (MP) on alloreactivity in mixed lymphocyte cultures and HLA-restricted host-directed cytotoxicity was investigated.[4] Since 1986 this method has allowed us to perform sixty haploidentical transplants (2-3 HLA loci mismatched) with bearable problems of GVHD and rejection. Furthermore it was used for marrow incubation in twenty patients with autoimmune diseases submitted to an autologous bone marrow transplantation (BMT).

We now aimed to verify whether this *in vitro* treatment resulted in a selective regulation of the Th1, Th2-like helper T-cell response. To evaluate this hypothesis we used an *in vitro* model to compare the cytokine production of T-cell subsets in response to phorbol myristate acetate in peripheral blood mononuclear cells (PBMC) cultures treated or not with VCR and MP.

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Design and Methods

Drug Treatment

For evaluation of intracellular cytokines, samples of heparinized whole blood from healthy volunteers (no. 7) or patients (no. 5) were treated with 1.5 μg/mL of vincristine alone (VCR), or 3 mg/mL of methylprednisolone alone (MP), or with a mixture of both drugs (VCR+MP). An untreated sample without drugs was also prepared as a negative control. All samples were incubated for 30 min a 37°C in a shaking waterbath. The cells were then washed twice in culture medium and used as described below. The viability of cells was also evaluated with the trypan blue exclusion method and was no less of 95% even after treatment with the drugs.

Staining for intracellular cytokines

The cells (treated and untreated) were stimulated for 4 h with 50 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma) and 10 mM ionomycin (Sigma) in the presence of 10 mM brefeldin A (Sigma), which blocks intracellular transport processes resulting in the accumulation of cytokine proteins in the Golgi complex.[5] Mononuclear cells were stained with peridinin chlorophyll protein (PerCP)-conjugated monoclonal antibody specific for the cell surface antigen CD3 (Caltag), then the cells were fixed by adding Reagent A (Fix&Perm, Caltag) for 15 minutes. The cells were washed twice with phosphate buffered saline (PBS) and were permeabilized by adding Reagent B (Fix&Perm, Caltag). These permeabilized cells were stained with FITC-labeled anti-human interferon (IFN)-γ monoclonal antibody and PE-labeled anti-human interleukin (IL)-4 monoclonal antibody (Caltag). Fluorochrome-conjugated, isotype-matched IgG1 and IgG2a were used as controls for detecting non-specific binding. After two washes with PBS, 500 μL of

the same buffer was added to all tubes and flow cytometric analysis was performed immediately.[6]

Cell culture: stimulation with PHA

Peripheral blood mononuclear cells (PBMC) from healthy volunteers and patients were purified by density gradient centrifugation with Ficoll-Hypaque , washed in Hank's balanced salt solution, and resuspended in RPMI 1640 media (Seromed SPA, Milan, Italy) supplemented with 10% of fetal calf serum (Seromed), 2 mM Glutamine and antibiotics. Cell density was adjusted to $0.5x10^6$ cells/mL and then treated as just described in the Drug Treatment section. Aliquots of treated or untreated samples were placed into sterile polystyrene round bottom tubes with caps, without stimulus or with three different concentrations of PHA 1, 3, 6 µg/mL.[7] Cells were cultured in a humidified incubator at 37°C in 5% CO_2.

Immunofluorescence staining for CD69 expression

Stimulated or unstimulated samples were harvested at 72h, washed twice in HBSS 1%BSA, and then 50 µL were put into each test tube and labeled with CD3 PerCP, CD69 PE and CD4 FITC (Caltag). Labeling was performed for 30 min a 4°C followed by a washing step. Samples were then resuspended in 500 µL of PBS and analyzed by flow cytometry.

Apoptosis detection

Apoptosis evaluation of cells treated with MP, VCR and MP + VCR was carried out using flow cytometric analysis.[8] Briefly, cells were pelleted at 200 g for 5 min., washed in PBS and resuspended in 200 µL of a binding buffer [10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl and 2.5 mM $CaCl_2$ (Bender MedSystem, Austria)]. Staining and analysis were carried out according manufacturer's instructions. Briefly, 5 µL of FITC-Annexin V (AnV final concentration: 1 mM) and 10mL of propidiun iodide (PI) (20 mg/mL) were added to each cell suspension. Cells were incubated at room temperature for 5-15 min in the dark and subsequently analyzed by flow cytometry. Annexin V fluorescence emission was detected in FL-1 (green fluorescence). PI staining is a dye-exclusion assay that discriminates between cells with intact membranes (PI-) and those with permeabilized membranes (PI+).

Flow cytometry

Samples were analyzed by three-color analysis using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Josè, CA, USA) equipped with an air-cooled argon ion laser. Ten thousands events were acquired in list mode and data analyzed with LYSIS II software. A gate was defined on the lymphocyte population on the basis of SSC/FSC properties; in addition a T-cell gate defined by SSC and Fl3 was used for $CD3^+$ cells. For all experimental conditions matched subclass controls were employed to determine the level of non-specific binding.

Results

The experiments performed on healthy controls show a marked down-regulation of IFN-γ and IL-4 intracellular production, indicating that *in vitro* drug treatment inhibited both the Th1 and Th2-like responses. Significantly lower number of $CD3^+$ T-cells producing IFN-γ and IL-4 were found in treated samples as compared to in untreated ones. MP alone caused a more marked decrease of cytokine production than VCR alone, but the effect was slightly enhanced by using the two drugs in combination (Figure 1). We also obtained similar results in patients submitted to allogeneic transplantation. Data, expressed as means ± SD for healthy controls and for a group of patients studied (Table 1), show that treatment with a combination of MP and VCR results in a significant decrease of CD3 producing IFN-γ ($p< 0.0005$) and IL-4 ($p <0.01$)

We demonstrated that this suppression was not associated with a cytotoxic effect of the drugs, because after treatment with the indicated doses of MP and VCR, alone or in combination, cells remained viable and there was no increase in their apoptosis (Table 2).

Indeed, the drug treatment did not even induce an impaired response to activation with PHA, as shown in Figure 2.

Discussion

Th1 and Th2 subsets of T-lymphocytes are involved in the pathogenesis of acute GVHD.[10] We demonstrated that by means of a short incubation with vincristine and methylprednisolone the activity of these cells can be paralyzed without interfering with cell viability. Of course we cannot know what will happen in the long run,

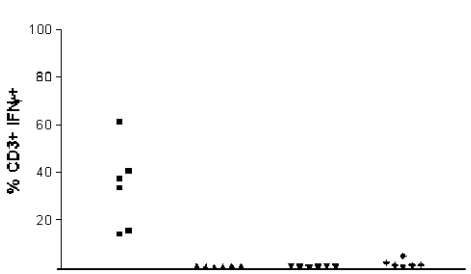

Figure 1.

Table 1. Percentage of peripheral blood CD3+ cells producing IFN-γ (Th1-like) and IL-4 (Th2-like) when untreated or treated with methylprednisolone and vincristine.

	% CD3 IFN-γ		% CD3	
	Untreated	Treated	Untreated	Treated
Controls (n=7)	34.69±15.43	0.18±0.14*	2.84±1.99	0.53±1.13°
Patients (n=5)	41.66± 6.80	0.17±0.04*	3.48±1.05	0.20±0.06°

Data are expressed as mean±standard deviation. p< 0.0005; ° p< 0.01.*

Table 2 . Effect of vincristine (VCR) and methylprednisolone (MP) on the staining of cells with annexin V-FITC (AnV) and propidium iodide (PI).

Cell characteristics	Untreated	Treated		
		Vincristine	MP	VCR + MP
% PI+ (dead)	0.01±0.006	0.01±0.02	0.00	0.01±0.005
% PI+ AnV+ (late apoptotic)	0.01±0.004	0.02±0.023	0.05±0.031	0.01±0.027
% AnV+ (early apoptotic)	0.55±0.045	0.78±0.049	1.23±0.235	0.81±0.12
% AnV-PI- (viable)	99.43±1.387	99.18±1.69	98.17±2.14	99.17±1.841

Values are expressed as percentage mean±SD of five different experiments.

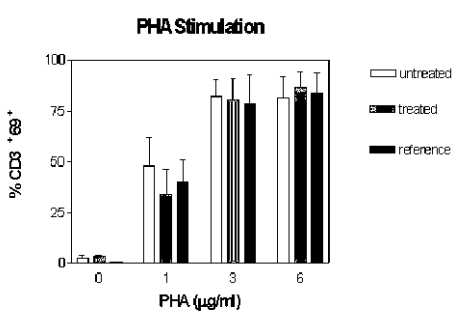

Figure 2.

but it seems likely that in some ways this transient paralysis favors the achievement of an antigen-specific immune tolerance. The clinical experience demonstrated that even in the worst situation, a 3 HLA mismatched BMT, GVHD becomes rather mild and in any case not worse than that occurring after a matched unrelated donor transplant. In two patients with advanced leukemia, 4-6 months after BMT we infused a few billion untreated donor's lymphocytes; in one patient a mild chronic GVHD occurred (the patient is a long-term survivor), in the other no GVHD developed at all (the patient died of relapse). It seems that the naive lymphocytes received a "message of tolerance" from the lymphocytes that acquired tolerance after our treatment and contact with the foreign HLA.

A transient immune blockade and a tolerance probably limited to the mismatched antigens that the donor's lymphocytes meet soon after the infusion into the recipient, would have the advantage of avoiding the long-lasting immune deficiency that patients, who receive a T-cell depleted marrow quite often experience.

We have no evidence that the same phenomenon occurs in the autologous setting, even though the clinical results in autoimmune diseases seem encouraging.

References

1. Hiscott A, McLellan DS. Graft-versus-host disease in allogeneic bone marrow transpalantation. the role of monoclonal antibodies in prevention and treatment. Br J Biomed Sci 2000; 57:163-9.
2. Fehse B, Goldmann M, Frerk O, Bulduk M, Zander AR. Depletion of alloreactive donor T cells using immunomagnetic cell selection. Bone Marrow Transplant 2000; 25:S39-42.
3. Fehse B, Frerk O, Goldmann M, Bulduk M, Zander AR. Efficient depletion of alloreactive donor T lymphocytes based on expression of two activation-induced antigens (CD25 and CD69). Br J Haematol 2000; 109:644-51.
4. Lanfranchi A, Andolina M, Tettoni K, et al. Functional depletion of T cells by vincristine and methylprednisolone as an in vitro model for the prevention of graft versus host disease. Haematologica 1992; 77:11-5.
5. Klausner RD, Donaldson JG, Lippincott-Schwartz J. Brefeldin A: insights into the control of membrane traffic and organelle structure. J Cell Biol 1992; 116: 1071-80.
6. Picker LJ, Singh MK, Zdreaveski Z, et al. Direct demonstration of cytokine synthesis heterogeneity among human memory/effector T cells by flow Cytometry. Blood 1995; 86:1408.
7. Maino VC, Sumi MA, Ruitenberg JJ. Rapid flow cytometric method for measuring lymphocytes subset activation. Cytometry 1995; 20:127-33.
8. Vermes I, Haanes C, Steffens-Wakken H, Revteleingsperger C. A novel assay for apoptosis. Flow cytometric detection of phospatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J Immunol Methods 1995; 184:39-51.
9. de Manzini A, Andolina M, Agosti E, et al. Low incidence of GvHD and rejection after pharmacological ex vivo modulation of bone marrow in 2-3 antigens mismatched BMT. Bone Marrow Transplant 1993; 11:114-6.
10. Nikolic B, Lee S, Bronson RT, Grusby MJ, Sykes M. Th1 and Th2 mediate acute graft-versus-host disease, each with distinct end-organ targets. J Clin Invest 2000; 105:1289-98.

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Autologous bone marrow transplantation versus alternative drugs in pediatric rheumatic diseases

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ABSTRACT

A minority of children suffering from severe rheumatic diseases are unresponsive to conventional treatments. These patients can now be managed with a variety of immunosuppressive therapies. Methotrexate is considered the first choice disease-modifying agent for adult and juvenile rheumatoid arthritis. In patients unresponsive to low doses of methotrexate, medium or high-doses can be useful. Instead of methotrexate, a recently develped immunosuppressive drug, mycophenolate-mofetil, which inhibits T- and B-lymphocyte proliferation, can be used. Another possibility for refractory rheumatic diseases, with no increase in toxicity, is combination therapy, for example methotrexate plus cyclosporine, or methotrexate plus salazopyrine or intravenous pulses of cyclophosphamide and methylprednisone. More recently two distinct inhibitors of tumor necrosis factor (etanercept and infliximab have been used successfully for intractable rheumatic diseases (juvenile idiopathic arthritis, psoriatic arthritis, spondyloarthropathies) but the follow-up is still too short to establish their long-term effectiveness. If all these treatments are unsuccessful, an autologous bone marrow transplantation can be proposed to selected patients. Interesting results have been obtained in pediatric rheumatic diseases such as juvenile idiopathic arthritis, systemic lupus erythematosus and systemic sclerosis. Further studies are required to assess the best procedures able to induce remission with a minimal risk of fatal events.

Key words: ABMT, pediatric rheumatic disease

Despite the overall prognosis being good for most patients suffering from pediatric rheumatic diseases, in a minority of children the disease is intractable, being unresponsive to non-steroidal anti-inflammatory drugs even in combination with steroids and/or immunosuppressive therapy.

Among the immunosuppressive drugs, methotrexate is increasingly being used not only in juvenile idiopathic arthritis (JIA) but also in psoriatic arthritis,[1] spondyloarthropathies,[2,3] systemic lupus erythematosus,[4] Crohn's disease[5] and also in chronic uveitis,[6] because of the quick reaction time to this drug and its superior effectiveness and tolerability. For these reasons, it is nowadays considered the first choice disease-modifying agent for adult and juvenile rheumatoid arthritis.

A collaborative study of the *Italian Pediatric Rheumatology Group*[7] demonstrated that at the conventional dose regimen methotrexate is effective in about 60% of patients suffering from JIA and is equally safe when administered orally or by intramuscular injections.

Some patients fail to achieve remission with the standard dosage (10 mg/m^2/weekly) but respond to higher dosages (from 15 to 20 mg/m^2/weekly). We do not know how many patients could benefit from medium and high doses (a *Pediatric Rheumatology International Trials Organization* study about this is ongoing), however this treatment represents another chance for such patients.

The clinical effectiveness of methotrexate in the treatment of resistant juvenile idiopathic arthritis was established in 1992[8] following a controlled short-term study.

More recently Ravelli *et al.*[9] have shown that methotrexate really has a "disease-modifying" potential in JIA, given its capacity to slow down the radiologic progression of the disease.

The drug is very safe and well tolerated and its long-term use does not appear to be associated with development of significant liver fibrosis even in patients who receive a cumulative dose equal to 3 g/m^2 of body surface area.[10,11]

Even though methotrexate represents an important step forward in the treatment of infantile rheumatic diseases, about 40% of treated patients do not respond in a satisfactory way and require other immmunosuppressive drugs.

Although there is little experience in the field of pediatric rheumatology,[12] another immunosuppressive drug that could be used when methotrexate fails, is mycophenolate mofetil, a

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pro-drug of mycophenolic acid, which inhibits purine biosynthesis and the proliferation of B- and T-lymphocytes. Clinical improvement has been seen in many mycophenolate mofetil-treated patients with rheumatoid arthritis who had been refractory to treatment with a variety of other disease-modifying anti-rheumatic drugs.[13]

The most frequent side-effects of mycophenolate mofetil are of gastrointestinal type. No clinically significant nephrotoxicity, hepatotoxicity or bone marrow toxicity attributable to this drug has been demonstrated.[13]

Another treatment possibility for refractory rheumatic diseases is combination therapy, for example methotrexate plus cyclosporine, or methotrexate plus salazopyrine plus steroids.

O'Dell *et al.*[14] demonstrated that a combination of methotrexate and hydroxychloroquine is more effective than methotrexate alone in adult rheumatoid arthritis and this enhanced effectiveness occurred with no increase in toxicity during the two years of treatment.

Tugwell *et al.*[15] showed that adults with severe rheumatoid arthritis and only partial response to methotrexate experienced a clinically important improvement after combination therapy with cyclosporine and methotrexate with substantially similar side-effects.

Wallace *et al.*[16] used monthly intravenous pulses of cyclophosphamide and methylprednisone to treat four patients with systemic-onset juvenile idiopathic arthritis that had remained active despite therapy with combination of methotrexate, sulfasalazine and hydroxychloroquine. Three patients achieved remission and all had an increase in linear growth. Pulsed cyclophosphamide could be added to methotrexate in severe, refractory JIA with growth retardation.

The benefits of pulsed compared with oral cyclophosphamide include: 1) lower total dose and risk of secondary malignancy; 2) ability to ensure adequate hydration and drugs that protect the bladder from hemorrhagic cystitis; 3) reduced risk of gonadal dysfunction.

In the last few years the pathogenetic studies on rheumatic inflammatory processes have brought to light new therapeutic possibilities for severe, intractable forms of JIA.

Tumor necrosis factor (TNF) is a pro-inflammmatory cytokine that has an important, complex role in the pathogenesis of rheumatic diseases. Two distinct inhibitors of this cytokine have recently been licensed in USA. Etanercept (Enbrel, Immmunex, Seattle), a genetically engineered fusion protein consisting of two identical chains of the recombinant TNF-receptor p75 monomer fused with the Fc domain of human IgG_1, binds and deactivates TNF[17] and infliximab (chimeric human-mouse anti TNF-alpha monoclonal antibody).[18]

Weinblat *et al.*[17] employed etanercept and methotrexate to treat a group of patients with adult rheumatoid arthritis not responsive to methotrexate alone in a double-blind trial. This combined therapy resulted in a rapid and sustained improvement and the only adverse effects associated with etanercept were mild injection-site reactions. More recently, Lovell *et al.*[19] used this drug in patients with polyarticular JIA who did not tolerate or had an inadequate response to methotrexate. In the first part of the study (open-label study) 74% of children had a good response to etanercept treatment. In the second part (double-blind study) 81% of cases who received placebo relapsed against 28% of patients who received etanercept. The drug was also effective in psoriatic arthritis.[20] A combination of infliximab plus methotrexate was also more effective than methotrexate alone in patients with active rheumatoid arthritis who have not previously responded to methotrexate.[18] This drug proved effective in an open-label pilot study in active spondyloarthropathy and in the treatment of Crohn's disease.[22]

Both these drugs may be able to induce remission in severe, non-responsive JIA. They are well tolerated and safe, but the follow-up in the different studies is too short to establish their ability to achieve long-term remission and to change the natural history of this disease, that should be considered the principal aim of this kind of therapy. Only a stable remission actually allows a resumption of growth, a reduction of osteoporosis and can put a stop to progressive articular damage.

In our opinion, only after having tried medium-high doses of methotrexate, in some cases associated with other immunosuppressive drugs and/or TNF-inhibitors, and when faced with an inadequate response or with a relapse within a short time, can it be justified to suggest a bone marrow transplantation (BMT).

Autologous hemopoietic stem cell transplantation has been proposed in a consensus report published by the *European League Against Rheumatism*[23] as a possible treatment for severe autoimmune diseases such as rheumatoid arthritis, systemic sclerosis, and systemic lupus erythematosus.

Only a few pediatric patients have undergone this treatment, so it is very difficult to decide when BMT can be proposed to children with intractable autoimmune diseases, especially in those with non-fatal inflammatory disorders.

The most important experience is that of Wulffraat who initially described a small series of four patients with refractory juvenile chronic arthritis[24] and more recently presented a series of 12

patients, 10 with a systemic form and 2 with a polyarticular form. The procedure entails T-cell depletion, a conditioning regimen with heavy immunosuppressive therapy and total body irradiation (TBI).[25] The results showed an aplastic period lasting 15-28 days and a marked decrease in arthritis severity as expressed in core set criteria for JIA activity.

However three children with active systemic features experienced an infection-associated hemophagocytic syndrome known as macrophage activation syndrome (MAS) which was fatal in all cases.[25]

The same author described two patients with severe juvenile SLE who achieved remission after autologous stem cell transplantation with the same procedure,[26] and Traynor *et al.* reported on seven patients free from signs of active lupus after high-dose chemotherapy and autologous hemopoietic stem-cell transplantation.[27]

Martini *et al.*[28] reported that autologous peripheral blood stem cell transplantation without TBI was safe and well tolerated in three patients with systemic sclerosis and progressive pulmonary disease resulting in marked and sustained clinical improvement in two of the three.

Our study protocol, described in this same issue by Rabusin *et al.*, does not entail myeloablation and involves a lighter conditioning regimen, but did bring about disease remission; however, the transitory nature of each remission means that the procedure cannot be credited with being responsible for changing the natural history of the diseases. Nonetheless the results are worthy of interest since any remission, however brief, should be considered a benefit for these patients.

In contrast, other procedures with myeloablation and/or TBI, in our opinion, entail too high a risk of fatal events in refractory, active systemic JIA suggesting the need for a more prudent policy in patients with non-fatal inflammatory disorders.

New strategies, possibly differentiated according to disease type and severity, with or without myeloablation, with or without TBI, should be planned by controlled studies in order to establish procedures able to induce remission with a minimal risk of fatal events.

References

1. Cuellar ML, Espinoza LR. Methotrexate use in psoriasis and psoriatic arthritis. Rheum Dis Clin North Am 1997; 23:797-809.
2. Cuellar ML, Espinoza LR. Management of spondyloarthropathies. Curr Opin Rheumatol 1996; 8:288-95.
3. Toussirot E, Wendling D. Use of methotrexate in spondyloarthropathies. Review of the literature. Rev Med Interne 1996; 17:333-9.
4. Walz LeBlanc BA, Dagenais P, Urowitz MB, Gladman DD. Methotrexate in systemic lupus erythematosus. J Rheumatol 1994; 21:836-8.
5. Feagan BG, Rochon J, Fedorak RN, et al. Methotrexate for the treatment of Crohn's Disease. N Engl J Med 1995; 332:292-7.
6. Weiss AH, Wallace CA, Sherry DD. Methotrexate for resistant chronic uveitis in children with juvenile rheumatoid arthritis. J Pediatr 1998; 133:266-8.
7. Ravelli A, Gerloni V, Corona F, et al. Oral versus intramuscular methotrexate in juvenile chronic arthritis. Clin Exp Rheum 1998; 16:181-3.
8. Giannini EH, Brewer EJ, Kuzmina N, et al. Methotrexate in resistant juvenile rheumatoid arthritis. Results of the USA-USSR double blind, placebo-controlled trial. N Engl J Med 1992; 326:1043-9.
9. Ravelli A, Viola S, Ramenghi B, et al. Radiologic progression in patients with juvenile chronic arthritis treated with methotrexate. J Pediatr 1998; 133:262-5.
10. Hashkes PJ, Balistreri WF, Bove KE, Ballard ET, Passo MH. The relationship of hepatotoxic risk factors and liver histology in methotrexate therapy for juvenile rheumatoid arthritis. J Pediatr 1999; 134:47-52.
11. Kugathasan S, Newman AJ, Dahms BB, Boyle JT. Liver biopsy findings in patients with juvenile rheumatoid arthritis receiving long-term, weekly methotrexate therapy. J Pediatr 1996; 128:149-51.
12. Gallagher KT, Bernstein B. Juvenile rheumatoid arthritis. Curr Opin Rheumatol 1999; 11:372-6.
13. Goldblum R. Therapy of rheumatoid arthritis with mycophenolate mofetil. Clin Exp Rheumatol 1993; 11 suppl 8:S117-9.
14. O'Dell JR, Haire CE, Erikson N, et al. Treatment of rheumatoid arthritis with methotrexate alone, sulfasalazine and hydroxychloroquine, or a combination of all three mediactions. N Engl J Med 1996; 334:1287-91.
15. Tugwell P, Pincus T, Yocum D, et al. Combination therapy with cyclosporine and methotrexate in severe rheumatoid arthritis. N Engl J Med 1995; 333:137-41.
16. Wallace CA, Sherry DD. Trial of intravenous pulse cyclophosphamide and methylprednisolone in the treatment of severe systemic onset juvenile rheumatoid arthritis. Arthr Rheum 1997; 40:1852-5.
17. Weinblatt ME, Kremer JM, Bankhurst AD, et al. A trial of etanercept, a recombinant tumor necrosis factor receptor:Fc fusion protein, in patients with rheumatoid arthritis receiving methotrexate. N Engl J Med 1999; 340:253-9.
18. Maini R, St Clair EW, Breedveld F, Furst D, Kalden J. Infliximab (chimeric anti-tumor necrosis factor a monoclonal antibody) versus placebo in rheumatoid arthritis patients receiving concomitant methotrexate: a randomised phase III trial. Lancet 1999; 354:1932-9.
19. Lovell DJ, Giannini EH, Reiff A, et al. Etanercept in children with polyarticular juvenile rheumatoid arthritis. Pediatric Rheumatology Collaborative Study Group. N Engl J Med 2000; 342:763-9.
20. Mease PJ, Goffe BS, Metz J, et al. Etanercept in the treatment of psoriatic arthritis and psoriasis: a randomised trial. Lancet 2000; 356:385-90.
21. Van der Bosch F, Kruithof E, Baeten D, et al. Effects of loading dose regimen of three infusions of chimeric monoclonal antibody to tumor necrosis factor a (infliximab) in spondyloarthropathy: an open pilot study. Ann Rheum Dis 2000; 59:428-33.
22. Hyams JS, Markowitz J, Wyllie R. Use of infliximab in the treatment of Crohn's disease in children and ado-

lescents. J Pediatr 2000; 137:192-6.
23. Tyndall A, Gratwohl A. Blood and marrow stem cell transplants in autoimmune disease: a consensus report written on behalf of the European League against Rheumatism (EULAR) and the European Group for Blood and Marrow Transplantation (EBMT). Bone Marrow Transplant 1997; 19:643-5.
24. Wulffraat N, van Royen A, Bierings M, Vossen J, Kuis W. Autologous haemopoietic stem-cell transplantation in four patients with refractory juvenile chronic arthritis. Lancet 1999; 353:550-3.
25. Brinkman DMC, Kamphuis S, Quartier P, et al. Infection associated MAS in three patients receiving ASCT for refractory JIA. Ann Rheum Dis 2000; 59:729-30.
26. Wulffraat NM, Vlieger AM, Kamphuis SM, Sanders EAM, Kuis W. Autologous stem cell transplantation in two patients with juvenile SLE. Ann Rheum Dis 2000; 59:744-5.
27. Traynor AE, Schroeder J, Rosa RM, et al. Treatment of severe systemic lupus erythematosus with high-dose chemotherapy and haemopoietic stem-cell transplantation: a phase I study. Lancet 2000; 356:701-7.
28. Martini A, Maccario R, Ravelli A, et al. Efficacy and safety of autologous peripheral blood stem cell transplantation in three children with systemic sclerosis and progressive pulmonary disease. Ann Rheum Dis 2000; 59:739-40.

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