Chimerism status is a useful predictor of relapse after allogeneic stem cell transplantation for acute leukemia

MANUEL BARRIOS, ANTONIO JIMÉNEZ-VELASCO, JOSÉ ROMÁN-GÓMEZ, MARÍA ELENA MADRIGAL, JUAN ANTONIO CASTILLEJO, ANTONIO TORRES, ANABEL HEINIGER

Background and Objectives. The role of hematopoietic chimerism after allogeneic stem cell transplantation (SCT) for acute leukemia remains controversial. We studied the relationship between hematopoietic chimerism and several prognostic variables on the outcome of SCT in patients with acute leukemia.

Design and Methods. Chimerism was determined by a semiquantitative method, based on polymerase chain reaction (PCR) amplification of variable number tandem repeat (VNTR) minisatellites, in 133 consecutive patients who underwent allogeneic SCT for acute leukemia (68 myeloid, 58 lymphoid and 7 biphenotypic), all receiving a myeloablative conditioning regimen.

Results. The median follow-up for the surviving patients was 44.8 months (range: 12.0-129.0). Recipient hematopoiesis (mixed chimerism, MC) was detected in 40 cases (30.1%). Two types of patients could be distinguished in this MC group: 29 with increasing MC and 9 with decreasing MC. The remaining 93 cases maintained complete donor chimerism (CC) over the whole follow-up period. Patients with increasing MC showed a significantly higher (p<0.001) rate of relapse (93.1%) and death (89.7%) in comparison to both those with CC (26.9% relapse, 44.1% dead) or decreasing MC (11.1% relapse, 44.4% dead). The detection of increasing MC preceded relapse by a median of 74 days (range: 5–434) and was significantly related with the absence of chronic graft-versus-host disease. Univariate and multivariate analysis confirmed that chimerism was the most significant variable involved in relapse, leukemia-free survival and overall survival after SCT.

Interpretation and Conclusions. These results demonstrate that sequential determination of chimerism allows the prediction of relapse and death after SCT for acute leukemia. The interval between detection of increasing MC and relapse may permit timely implementation of therapeutic measures.

Key words: hematopoietic chimerism, stem cell transplantation, acute leukemia, VNTR, PCR.

Haematologica 2003; 88:801-810
http://www.haematologica.org/2003_07/801.htm

From the Hematology Department, Carlos Haya Hospital, Málaga (MB, AJ-V, AH), Hematology Department, Reina Sofía Hospital, Córdoba (JR-G, MEM, JAC, AT), Spain.

Correspondence: Antonio Jiménez-Velasco, MD, Hematology Department, Carlos Haya Hospital, Avda Carlos Haya, 29010 Málaga, Spain.
E-mail: antoniof.jimenez.sspa@juntadeandalucia.es

The development of allogeneic stem cell transplantation (SCT) as an efficient therapy for hematologic malignancies and the persistence of relapse of the underlying disease as its most important complication have stimulated the search for methods to monitor graft status and to anticipate morphologic relapse. This would then enable early tapering of immunosuppression or the administration of a rescue treatment, such as donor leukocyte infusions (DLI), even before the diagnosis of relapse has been established.[1] Chimerism techniques can detect the presence of recipient cells after SCT and allow the relative proportions of host and donor cells to be identified and quantified.[1] Minisatellites are highly polymorphic regions with a variable number of tandem repeats (VNTR) of nucleotides, located throughout the genome. Polymerase chain reaction (PCR) amplification of mini or microsatellite regions is one of the most sensitive and rapid methods of determining chimerism, and can be used even when very few cells are available for analysis.[2]

With regard to the outcome of allogeneic SCT, complete chimerism (CC) is usually accepted to be associated with a low risk of relapse and a better prognosis. However, the importance of residual host cells observed after SCT (mixed chimerism, MC) remains unclear. There are several reasons for this: first, the frequent presence of residual recipient cells soon after SCT which later disappear.[3] Second, differences in the sensitivity of the methods employed to study chimerism; indeed, MC is becoming more frequent as more sensitive detection techniques are developed.[4,5] Thirdly, a great number of variables seem to influence the appearance of MC; for example, the conditioning regimen,[6] the depletion of T cells,[7] the number of stem cells infused,[8] and the time interval between SCT and VNTR measurement,[2,9] have all been reported to affect the degree of MC. In patients receiving allogeneic SCT for chronic myeloid leukemia (CML), our group and others have demonstrated the value of chimerism follow-up combined with BCR-ABL determination to predict relapse.[10–12] However, these results in CML have not yet been confirmed in patients with acute leukemia, for whom an early detection of relapse is more important. Bader *et al.*,[13,14] using VNTR PCR amplification techniques, showed that the risk of relapse in children after allo-SCT was significantly enhanced when MC was present if samples were collected at sufficiently short time intervals. The predictive properties of MC had already been reported,[15] and other groups have recently confirmed these results using the same method.[16,17] More complex studies have also demonstrated a higher risk of relapse in patients

with lineage-specific MC.[18] Different results, however, were found in early chimerism studies[6,19] in which mixed T-lymphoid chimerism did not correlate with relapse but correlated with the host's age or the conditioning regimen. And later, using either *in situ* hybridization techniques[20] or short tandem repeat amplification,[21,22] chimerism status was not considered relevant for the outcome of patients with acute leukemia who underwent allogeneic SCT.

The aim of the present study was to clarify the role of MC, analyzed by VNTR amplification, in the prediction of relapse after allogeneic SCT for acute leukemia in a large series of patients with a long-term follow-up. The semiquantitive method employed allows the separation of patients with decreasing MC, who progressively recover full donor hematopoiesis, from those with increasing MC, in order to compare both groups with the CC group.

Design and Methods

Patients' characteristics

The charts of 147 consecutive patients with acute leukemia who received allogeneic SCT between January 1992 and December 2001 in two different Spanish Hospitals (Carlos Haya Hospital in Málaga and Reina Sofía Hospital in Córdoba) were reviewed. The last date for evaluation was 30 September 2002. Informed consent was obtained according to institutional guidelines. All patients received myeloablative conditioning regimens. Seven cases (4.8%) could not be included in the study because of a missing pre-transplant DNA recipient sample (n=3) or the absence of informative chimerism alleles (n=4). Of the remaining 140 patients, 5 were excluded because of early mortality without leukocyte implant after SCT and 2 because no evidence of complete remission could be assessed after SCT; chimerism determination in these cases is of no benefit. The characteristics of the 133 patients included in the final analysis (age, sex, leukemia type, pre-SCT status) and the details of SCT procedures (donor type, conditioning regimen, product manipulation, and graft-versus-host disease prophylaxis) are shown in Table 1.

Table 1. Summary of patients' characteristics and SCT procedures.

	N (%)
Number of patients	133
Sex (M/F)	73/60
Age median yrs (range)	26.0 (2 - 55)
Leukemic lineage	
Myeloid	68 (51.5%)
Lymphoid	58 (43.6%)
Biphenotypic	7 (5.3 %)
Clinical status at SCT	
1st CR[a]	74 (55.6%)
2nd CR[a]	39 (29.3%)
>2nd CR[a] /refractory	20 (15.0%)
Donor type	
HLA-identical sibling	111 (83.5 %)
Matched unrelated	17 (12.8 %)
Haploidentical	5 (3.8 %)
Stem cell source	
Bone marrow	102 (76.7 %)
Peripheral blood	30 (22.6 %)
Umbilical cord blood	1 (0.8 %)
Conditioning regimen	
TBI[b] + chemotherapy[c]	78 (58.6%)
TBI[b]+ haploidentical[d]	5 (3.8%)
BUCY[e]± other drugs[f]	48 (36.1 %)
Other	2 (1.5 %)
T-cell depletion	
Yes	25 (18.8 %)
No	108 (81.2 %)
GVHD prophylaxis	
Cyclosporine A + Methotrexate	111 (83.5 %)
Cyclosporine A	17 (12.8 %)
Other	5 (3.8 %)

[a]CR: complete remission; [b]TBI, total body irradiation; [c]chemotherapy plus TBI; cyclophosphamide (71 cases), etoposide (5) or melphalan (1); [d]haploidentical chemotherapy includes antithymocyte globulin (ATG), fludarabine and thiotepa; [e]BUCY, busulphan + cyclophosphamide; [f]etoposide (7 cases).

Sample collection

Whole peripheral blood samples (3-5 mL) were collected for DNA extraction from both donor and recipient before transplantation in order to obtain an informative locus. During the first year after SCT, whole peripheral blood was collected monthly and bone marrow samples every 3 months. During the second year, the frequency was reduced to every 3 months for peripheral blood and every 6 months for bone marrow samples; thereafter, only peripheral blood samples were taken every 3-6 months. If the clinical situation warranted, more frequent chimerism evaluations or bone marrow aspirates were performed. Determinations of chimerism were recorded for this study until relapse or death.

DNA extraction

Genomic DNA was extracted from whole fresh blood or bone marrow collected in EDTA Vacutainer tubes, by detergent lysis, salt fractionation and isopropanol precipitation (Puregene DNA isolation kit, Gentra Systems, Minneapolis, MN, USA). The correct concentration and purity of each sample was confirmed by UV spectrophotometer at 260 and 280 nm. DNA samples were conserved at -80°C until PCR experiments were performed.

Chimerism analysis by VNTR polymerase chain reaction

Chimerism analysis was performed by amplification of VNTR sequences with PCR methods. Briefly,

a panel of six VNTR minisatellite loci (D1S80, APO-B, DXS52, 33-6, 33-1, YNZ22) was used to identify an informative marker between donor and recipient. The characteristics and sequences of the different primers used have been described previously.[23-25] All primers were obtained from Amersham Biosciences Europe GmbH (Freiburg, Germany) and purified by high-performance liquid chromatography. The final volume for PCR amplification was always 50 μL, containing about 500 ng DNA, 10 mM Tris-HCl buffer (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM dNTPs (Roche Diagnostics GmbH, Mannheim, Germany), 1 mM of each primer and 2.5 U of Taq DNA polymerase (Roche Diagnostics). The denaturation, annealing and extension cycles were programmed in a Perkin-Elmer 2400 thermal cycler (Norwalk, CT) as previously described for each locus.[23-25] All reactions finished with an extension time of 10-20 min at 72°C, followed by a reduction to 4°C for conservation. The PCR amplification products were then separated by 1.5% agarose gel electrophoresis and visualized under UV-light stimulation by ethidium bromide (1 μg/mL) staining. The resulting images were digitalized and analyzed densitometrically by specific software (Bio-Profil Bio-1D, Viber Lourmat, Marne-La-Vallée, France). All samples were analyzed in at least two different PCR experiments.

In order to obtain a quantitative estimation of the MC, dilution experiments with host and donor DNA were performed and a standard curve was established for each patient as previously described,[10,14] with the aim of relating densitometric measures (peak areas) to DNA amount. Data were finally expressed as a percentage of the recipient DNA as shown in Figure 1. The sensitivity of this approach ranged from 1% to 2.5% depending on the marker and the different lengths of host and donor alleles.

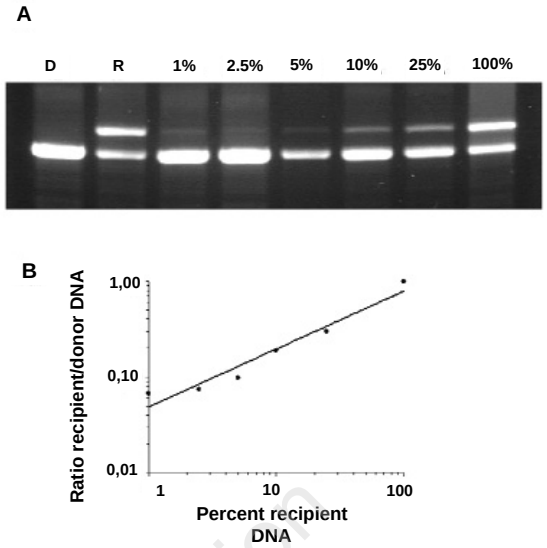

Figure 1. Typical dilutional experiment of recipient DNA with donor DNA. (a) Agarose gel electrophoresis after amplification of D1S80 VNTR in donor (D), recipient (R) and successive dilutions of the recipient is shown in the different lanes. (b) The regression line represents the amount of DNA versus the densitometric R/D ratio of each dilution, which is the procedure used for mixed chimerism estimations in each patient.

Definition of mixed chimerism, remission and relapse

Patients who showed no evidence of host DNA at any time during the post-transplant follow-up were considered to be in complete chimerism (CC). Patients with both donor and recipient DNA in any of the samples analyzed were defined as having mixed chimerism (MC). Two groups were distinguished in MC patients, according to Bader *et al.*,[14] with slight modifications. First, those with increasing levels of recipient DNA (either a 5% or greater increase between two successive chimerism assays, or those changing from CC to any level of MC), were referred to as having increasing MC. Second, those with a decreasing recipient DNA content (5% or greater decrease between two successive samples, or transformation from MC to CC) were referred to as decreasing MC.

In addition to those aspirates planned for the assessment of chimerism, bone marrow studies based on clinical criteria were also performed to evaluate disease status. Complete remission was confirmed when fewer than 5% blast cells were found in regenerated bone marrow. A relapse was diagnosed when more than 5% blast cells were observed in bone marrow, or extramedullary leukemic cells appeared. Determinations of MC in samples collected at the time of relapse were not considered for analysis and at least one bone marrow in remission was always required in patients with MC.

Assessment of graft-versus-host disease (GVHD)

Acute GVHD was classified from grade 0 to grade 4 according to criteria previously described by Glucksberg *et al.*[26] Chronic GVHD was graded as none, limited or extensive according to Shulman *et al.*[27] and was only recorded in patients who were alive and in remission beyond day +100 from transplantation.

Statistical analysis

Differences in clinical variables were studied by the χ^2 test or Fisher's exact test for categorical data and by the non-parametric Kruskal-Wallis test for continuous data. A probability value (p) below 0.05 was considered statistically significant. The actuarial probabilities of relapse, overall survival (OS) and leukemia-free survival (LFS) were estimated by the

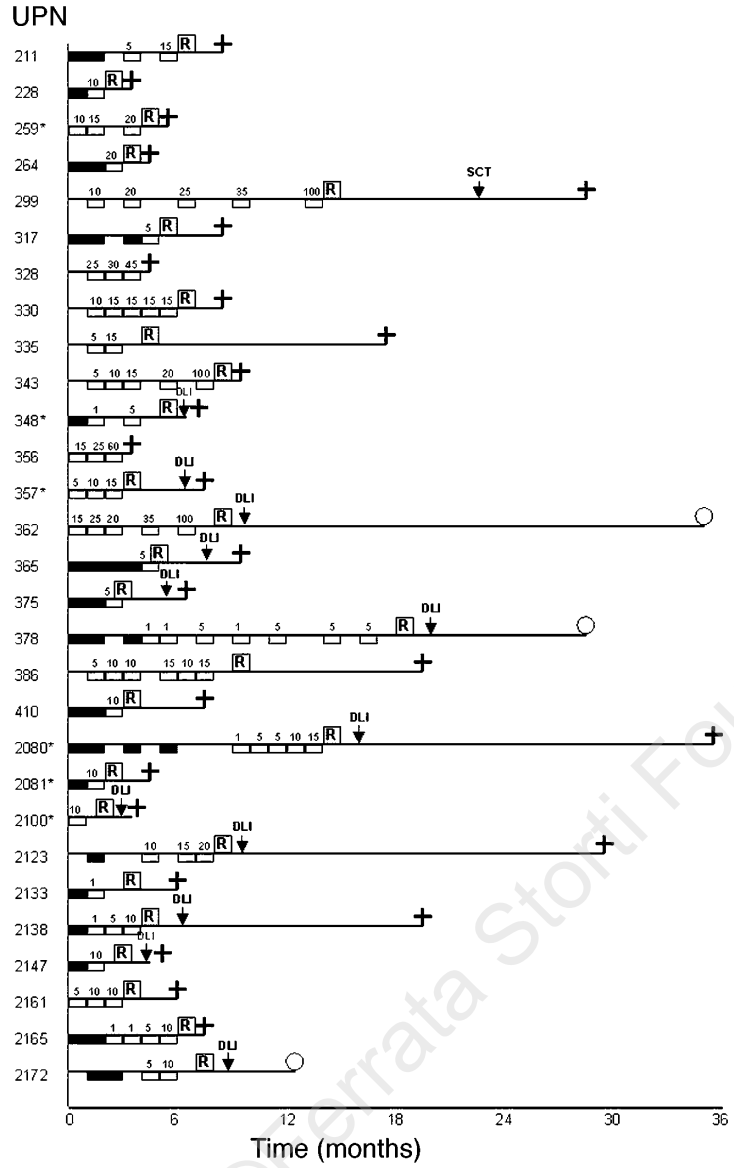

Figure 2. Course of patients with increasing mixed chimerism (iMC) after SCT. Each patient is represented by a horizontal line, with chimerism determinations depicted as squares below the line. Black squares indicate complete donor chimerism; grey squares indicate mixed chimerism; and white squares represent autologous recovery. The numbers above the lines represent the percentage of recipient chimerism found at each point. At the end of the lines, a cross indicates death and a blank circle indicates that the patient was still alive. R inside a square above the line indicates relapse. UPNs with an asterisk represent patients with active disease at the time of SCT. In order to simplify the presentation, no further chimerism samples are included once relapse was diagnosed.

Kaplan-Meier method. The endpoints for LFS curves were either death or relapse; the time to relapse was calculated by censoring dead patients without relapse and disease-free patients at the time of analysis. Comparisons of survival curves between chimerism groups were analyzed by the log rank test. A landmark approach, fixing the status of time-dependent variables (including GVHD or chimerism) at 120 days, was used to implement survival calculations, in order to minimize the bias due to the time-dependent character of chimerism. The selection of this cut-off point was based on the fact that by this point most prognostic factors (including chronic GVHD) have already reached their final values, with a minor loss in the number of patients. Uni- and multivariate analyses were performed in all patients for the whole follow-up period using a Cox proportional hazard regression model. In the case of time-dependent variables, a modification of the Cox regression for time-dependent covariates was employed to analyze the risk. The risk estimations were represented as odds ratios (OR). Only variables with $p < 0.10$ in the univariate analysis were included in the multivariate analysis.

Results

Post-SCT chimerism status

The median follow-up for living patients was 44.8 months (range: 12.0-129.0). Graft failure occurred in 2 patients, both of whom died within the first 6 months after transplantation. Relapse was found in

53 patients (39.8%), four presenting with extramedullary disease. In 18 of these relapsed patients, rescue treatment with DLI or a second SCT (in one case) was undertaken. Seventy-one patients died (53.4%), most because of progression of the leukemic disease (44 patients).

Ninety-three patients (69.9%) remained in CC for the whole follow-up period, whereas MC was detected at least once in the remaining 40 cases (30.1%). Of this MC group, 29 patients (21.8%) showed increasing MC, whereas in another 9, the MC decreased until CC was reached. Two T-cell depleted cases, whose MC remained stable during the whole follow-up period, could not be classified as having either increasing MC or decreasing MC.

Mixed chimerism and SCT outcome

Increasing mixed chimerism. There were 27 relapses (93.1%) in the 29 patients with increasing MC; the other 2 patients (UPN 328 and 356) died during the first 4 months after SCT due to secondary graft failure, as depicted in Figure 2. In some cases (12 out of 29), increasing MC appeared after the first chimerism determinations, whereas in the others chimerism changed from CC to increasing MC during the course of follow-up; however, the distribution of other prognostic variables and the patients' outcome were similar in both patterns of increasing MC (*data not shown*). The median time interval between the detection of MC and relapse was 74 days (range: 5 - 434), with 70% of patients showing MC at least one month before relapse. Regarding the CC group, relapse occurred in 25 of the 93 cases (26.9%), significantly fewer than in the increasing MC group (p<001, Fisher's exact test). Four of these 25 had the only extramedullary relapse found.

Increasing MC was also associated with significantly higher mortality than CC (89.7% vs. 44.1%; p<0.001, Fisher's exact test). Furthermore, in the CC group, the cause of death was unrelated to the primary disease in half the cases, whereas in the group with increasing MC, 84.0% of deaths were secondary to leukemic progression. Of note was the fact that, although most patients with increasing MC died because of leukemic progression, long survival (more than 6 months) after relapse was observed in 8 cases, as shown in Figure 2. Six of these patients received treatment with chemotherapy plus DLI or a second SCT after relapse, which resulted in increased survival: three (UPNs 362, 378 and 2123) recovered CC, the first 2 remaining alive with extensive chronic GVHD at the end of the recorded period, the third dying because of infectious complications and extensive GVHD. The fourth patient (UPN 299) received a second transplantation nine months after relapse, and then died of disease progression. Patients 5 and 6 (UPNs 2080 and 2138) reached temporary CC, but then new relapses refractory to treatment led to their death. Finally, patients 7 and 8

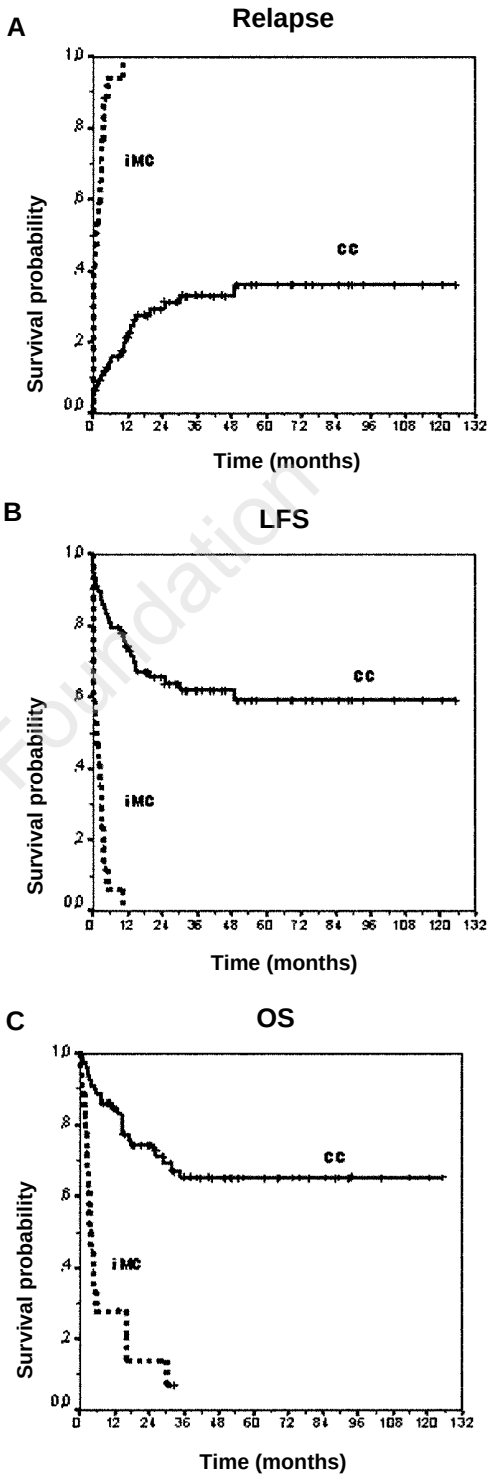

Figure 3. Relapse probability (a), leukemia-free survival (b), and overall survival (c) in the complete donor chimerism (CC) and the increasing mixed chimerism (iMC) groups, estimated by the Kaplan-Meier model at a landmark of 120 days. Comparisons between both groups were made with the log-rank test. (p < 0.001 in all cases).

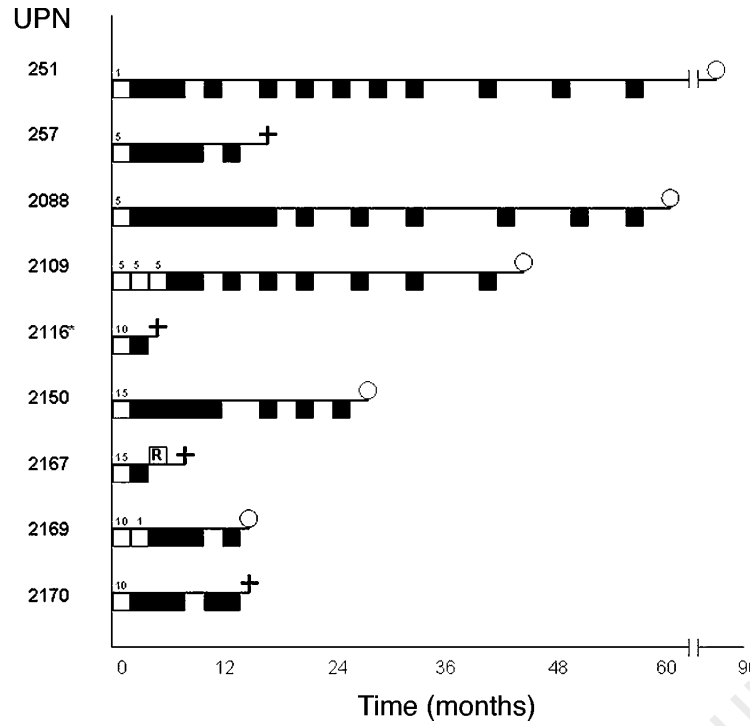

Figure 4. Course of patients with decreasing mixed chimerism (dMC) after SCT. Each patient is represented by a horizontal line, with chimerism determinations depicted as squares below the line. Black squares indicate complete donor chimerism and grey squares indicate mixed chimerism. The numbers above the lines represent the percentage of recipient chimerism found at each point. At the end of the lines, a cross indicates death, and a blank circle indicates that the patient was still alive. R inside a square above the line indicates relapse. UPNs with an asterisk represent patients with active disease at the time of SCT.

(UPNs 335 and 386) received only chemotherapy before dying of disease progression.

The curves for the probability of relapse, LFS and OS, calculated with a landmark at 120 days to minimize time-dependent bias, are illustrated in Figure 3. The probability of relapse was 100% in the group with increasing MC vs 36.0% in the group with CC (95% CI: 23.9-48.2). Mean LFS was 2.1 months (0.8-3.4) for those with increasing MC compared to 78.6 months (65.2-91.9) for those with CC ($p<0.0001$). OS was also significantly shorter in patients with increasing MC, 8.7 months (3.9-13.5) compared to 86.4 months (73.7-99.1) in those with CC ($p<0.0001$).

Decreasing mixed chimerism

The patients with decreasing MC rarely relapsed (1 of 9 patients, 11.1%, Figure 4) and their results did not differ statistically from the CC patients as regards frequency of relapse (11.1% vs 26.9% respectively; $p>0.1$, Fisher's exact test) or mortality (44.4% vs 44.1%; $p>0.1$). In most cases (3 of the 4), death in the decreasing MC group occurred early post-SCT, and was due to complications unrelated to their disease (severe GVHD in 2 cases -UPNs 2116 and 2160- and brain hemorrhage in the other -UPN 257); the remaining patient (UPN 2167) died of disease progression. This group had the lowest probability of relapse (12.5%; 95% CI: 0-34.4), its mean LFS was 52.7 months (95% CI: 27.1-82.4), and the mean OS was 54.9 months (95% CI: 27.5-82.4). Neither LFS nor OS was significantly different from the survival of the patients with CC ($p>0.1$).

Chimerism and other prognostic variables

With regard to the distribution of the patients at the selected landmark, at which time most patients had already reached their definitive values in time-dependent variables (such as chimerism or GVHD), no significant differences were found between the three chimerism groups in the distribution of most of the prognostic variables analyzed (Table 2), indicating that the differences in outcome cannot be attributed to other confounding variables. Three exceptions were noted: the use of radiotherapy for the conditioning regimen, which was less frequent in the decreasing MC group than in the other two groups ($p=0.021$, χ^2 test); the depletion of T-cells in the infusion product, more frequent in both mixed chimerism groups ($p=0.01$, χ^2 test); and the development of chronic GVHD, which was less common in the patients with increasing MC ($p=0.026$, χ^2 test).

Univariate Cox analysis of the prognostic variables that might predict post-SCT relapse (Table 3) showed that non-myeloid lineage, active pre-SCT disease, absence of acute or chronic GVHD, and increasing MC were significantly associated with a higher probability of relapse. The multivariate Cox model (Table 4) showed chimerism status and pre-SCT clinical status to be the only significant risk factors implicated in relapse.

Univariate analysis showed that the risk factors for LFS and OS were similar: older age, a haploidentical donor and the conditioning regimen given in haploidentical SCT, and a T-cell depleted transplant, as well as three of the main variables predicting

Table 2. Distribution of patients at the 120-day landmark in the groups analyzed, according to the main prognostic pre- and post-SCT variables.

	CC	*Increasing MC*	*Decreasing MC*	*p*
Number of patients	78	18	8	
Sex (M/F)	42/36	12/6	3/5	N.S.[1]
Age yrs (median)	23.5	20	26.5	N.S.[2]
< 15 yrs	18 (23%)	6 (33%)	3 (38%)	N.S.[1]
16-35 yrs	45 (58%)	7 (39%)	2 (25%)	
>35 yrs	15 (19%)	5 (28%)	3 (38%)	
Leukemic lineage				
Myeloid	40 (51%)	4 (22%)	6 (75%)	N.S.[1]
Lymphoid	34 (44%)	12 (67%)	2 (25%)	
Biphenotypic	4 (5%)	2 (11%)		
Clinical status at SCT				
1st-2nd CR[a]	71 (91%)	15 (83%)	7 (88%)	N.S.[1]
>2nd CR[a]/refractory	7 (9%)	3 (17%)	1 (13%)	
Donor type				
HLA-identical sibling	65 (83%)	14 (78%)	7 (88%)	N.S.[1]
Matched unrelated	12 (15%)	2 (11%)	1 (13%)	
Haploidentical	1 (1%)	2 (11%)		
Stem cell source				
Bone marrow	60 (77%)	14 (78%)	5 (63%)	N.S.[1]
Peripheral blood	18 (23%)	3 (17%)	3 (38%)	
Conditioning regimen				
TBIb + chemotherapy[c]	53 (68%)	10 (56%)	2 (25%)	0.021[1]
BUCYe± other drugs[f]	24 (31%)	6 (33%)	6 (75%)	
TBIb+ haploidentical[d]	1 (1%)	2 (11%)		
T-cell depletion	6 (8%)	6 (33%)	2 (25%)	0.010[1]
GVHD prophylaxis				
Cyclosporine A + MTX[g]	69 (89%)	14 (78%)	7 (88%)	N.S.[1]
Cyclosporine A	8 (10%)	2 (11%)	1 (13%)	
Other	1 (1%)	2 (11%)		
Acute GVHD (II-IV)	37 (47%)	5 (28%)	5 (63%)	N.S.[1]
Chronic GVHD	14 (18%)	1 (6%)	4 (50%)	0.026[1]

[a]CR: complete remission; [b]TBI: total body irradiation; [c]cyclophosphamide: (71 cases), etoposide (5) or melphalan (1); [d]Haploidentical chemotherapy includes antithymocyte globulin (ATG), fludarabine and thiotepa; [e]BUCY, busulphan + cyclophosphamide; [f]mainly etoposide; [g]methotrexate. Comparisons between the three chimerism groups: [1]χ^2 test; [2]Kruskal-Wallis test. NS, not significant.

Table 3. Univariate Cox analysis of prognostic variables for the probability of relapse, leukemia-free survival (LFS) or overall survival (OS) after SCT.

	Relapse		LFS		OS	
	OR[a]	p	OR[a]	p	OR[a]	p
Sex						
Male	1.03	N.S.	0.97	N.S.	0.90	N.S.
Age						
<15 yrs	0.99	N.S.	1.05	N.S.	1.08	N.S.
>35 yrs	1.27	N.S.	1.98	0.009	2.22	0.003
Leukemic lineage						
Non myeloid	1.88	0.032	1.34	N.S.	1.19	N.S.
Clinical status SCT						
Active disease	3.11	0.001	2.99	< 0.001	3.50	<0.00
Donor type						
Unrelated matched	0.69	N.S.	1.00	N.S.	1.27	N.S.
Haploidentical	3.10	0.063	3.33	0.011	4.30	0.002
Stem cell source						
Other than BM	1.07	N.S.	1.10	N.S.	0.97	N.S.
Conditioning regimen						
TBI [b]+ CMT[c]	1.15	N.S.	0.86	N.S.	0.72	N.S.
CMT[c] alone	0.87	N.S.	1.14	N.S.	1.38	N.S.
Haploidentical[d]	3.06	0.068	3.60	0.008	4.97	0.001
T-cell depletion						
Yes	1.62	N.S.	2.08	0.005	2.15	0.005
GVHD prophylaxis						
No CyA+Mtxe	0.85	N.S.	1.14	N.S.	1.32	N.S.
Acute GVHD (II-IV)						
Yes	0.54	0.034	0.64	0.064	0.68	N.S.
Chronic GVHD						
Yes	0.41	0.050	0.45	0.044	0.39	0.023
Chimerism status						
Increasing MC	13.14	<0.001	7.26	<0.001	3.89	<0.001
Decreasing MC	0.05	N.S.	0.886	N.S.	1.04	N.S.

[a]Data are expressed as the odds ratio (OR) estimated by the model with respect to the reference group; [b]TBI, total body irradiation; [c]CMT, chemotherapy; [d]includes TBI, antithymocyte globulin (ATG), fludarabine and thiotepa; [e]CyA+Mtx, cyclosporine A + methotrexate; N.S., not significant.

relapse: active disease, absence of chronic GVHD and increasing MC (Table 3). In the multivariate Cox analysis including time-dependent variables, chimerism and pre-SCT clinical status again represented the most powerful predictors of LFS and OS, even though older age and absence of chronic GVHD were also significant risk factors (Table 4).

Discussion

Relapse of the underlying disease continues to be the main cause of failure of allogeneic SCT in acute leukemia.[28,29] The figures differ depending on the variables considered, but up to 60% of high-risk patients with advanced disease may relapse after transplantation.[29] Early detection of relapse has been associated with a better response to therapeutic measures,[30] so that timely prediction of relapse could improve the overall results of allogeneic SCT. Although hematopoietic chimerism as a predictor of relapse becomes particularly relevant after transplantation for acute leukemia, conclusive studies have generally referred just to CML,[10,11,31] with its role in acute leukemia still being controversial.[6,19,21,32]

This study demonstrates three important points: the significant relation between increasing MC and the high risk of relapse and death in acute leukemia; the possibility of predicting relapse before this latter is confirmed morphologically; and the higher predictive value of sequential rather than isolated determinations of chimerism because of the heterogeneous character of MC.

Table 4. Results of multivariate analysis for the probability of relapse and overall survival (OS) after SCT using the Cox proportional hazard regression model adjusted for time-dependent covariates.

	Relapse		*LFS*		*OS*	
	OR[a]	*p*	*OR*[a]	*p*	*OR*[a]	*p*
Age (>35 yrs)	–	–	1.94 (1.12-3.38)	0.019	1.99 (1.15-3.46)	0.014
Non-myeloid lineage	1.48 (0.79-2.70)	N.S.	–	–	–	–
Active disease at SCT	2.74 (1.23-6.10)	0.014	2.16 (1.13-4.13)	0.020	2.92 (1.54-5.55)	0.001
Haploidentical donor[b]	0.39 (0.09-1.60)	N.S.	0.63 (0.20-2.01)	N.S.	0.87 (0.27-2.73)	N.S.
T-cell depletion	–	–	0.96 (0.48-1.91)	N.S.	1.22 (0.63-2.37)	N.S.
Acute GVHD (II-IV)	0.55 (0.29-1.05)	0.071	0.74 (0.45-1.23)	N.S.	–	–
Chronic GVHD	0.30 (0.07-1.30)	N.S.	0.34 (0.12-0.98)	0.046	0.34 (0.12-0.97)	0.044
Increasing MC	10.47 (5.45-20.1)	<0.0001	5.91 (3.35-10.4)	<0.0001	2.53 (1.41-4.56)	0.002

[a]Data are expressed as the odds ratio (OR) estimated by the model with respect to the reference value; [b]Haploidentical donor type and haploidentical conditioning regimen are considered the same variable because of their covariance.

Our results confirm that the appearance of increasing amounts of recipient cells after SCT (increasing MC), detected by semiquantitative PCR amplification of VNTR loci, is followed in most cases by relapse of acute leukemia. These results are in agreement with previously reported data, for which MC is of value in predicting relapse after SCT in acute leukemia.[13,14,18,33,34] In common with reports of chimerism as a predictor of outcome, our work shares the same method for determining chimerism: amplification of VNTR by PCR. The high frequency and polymorphism of VNTR enables an informative locus to be found in about 95% of patients, and this PCR amplification is generally accepted as one of the most sensitive methods in the field.[1,2,35] Other methods, such as conventional cytogenetics[7] or erythrocyte markers,[36] failed to detect an association between chimerism and outcome in acute leukemia.

Our data show that patients with MC do not form a homogeneous group, so that MC as such can represent either a positive or a negative prognostic factor, depending on serial determinations. Grouping patients into those with increasing and those with decreasing MC is a common feature of previous studies showing a predictive value for chimerism.[10,14,18] The semiquantitative properties of the method employed allow this simple but useful classification, since MC patients can behave very differently; in fact, the outcome of patients with decreasing MC did not differ from that of patients with CC, whereas subjects with increasing MC had a high probability of relapse and death. The inclusion of patients with both decreasing and increasing MC in a single group in some studies could have masked the recognition of chimerism as a risk factor for SCT outcome.[21]

Although univariate analysis showed that several variables have a significant influence on SCT outcome, the high predictive value of chimerism, as determined in the present work, is more prominent when submitted with the other prognostic variables to multivariate analysis. Chimerism was the most powerful variable to explain the probability of relapse, and both leukemia-free survival and overall survival. Other important risk factors, such as age, pre-SCT situation or GVHD, lost part of their statistical significance in the presence of chimerism status.

Only three of the variables recorded show an unbalanced distribution in the chimerism groups and so could have influenced the outcome analysis. The conditioning regimen with radiotherapy (less frequent in the group with decreasing MC), and T-cell depletion of the infusion product (more frequent in both MC groups), have both been described before.[36,37] The greater myeloablative effect of total body irradiation may reduce the number of recipient residual cells after SCT;[6,34,36] some of the patients who would otherwise have been included in the decreasing MC group therefore reverted directly to the CC group. With regard to T-cell depletion, this has been associated with tolerance to the donor implant and the presence of long-term mixed chimerism.[37-39] However, none of these variables was significant risk factors and their distribution failed to correlate with outcome.

Chronic GVHD was the only significant risk factor for outcome (in multivariate LFS and OS) associated with chimerism status. It was more frequent in those groups with better outcome (CC and decreasing MC) and was almost absent in the group with increasing MC. The protective effect of chronic GVHD has repeatedly been described and is based on its relation with the graft-versus-leukemia effect of donor cells.[40,41] It should be emphasized that detection of recipient cells by chimerism mostly anticipated relapse by at least two months. A similar delay between chimerism and relapse has been observed in other studies[18,42] and this may be enough to begin therapeutic measures, such as tapering immunosuppression or DLI. The efficacy of these rescue treatments has been thoroughly demonstrated[33,43,-45]

Finally, although useful for predicting relapse, chimerism status is not wholly predictive; in our series, about 25% of patients (25 out of 93) suffered a relapse shortly after demonstrating complete donor chimerism. This situation has been reported elsewhere.[13,18,34] The time schedule, previously suggested as an explanation for these cases, cannot be

applied to our study, since most of the relapsed patients had had a CC sample in the two months prior to their relapse. Although the four patients with extramedullary relapse belonged to the CC group, relapse still appeared in a number of other patients with no change in chimerism status. In these cases, a possible explanation could reside in the sensitivity of present methods. Promising results have recently been reported using quantitative real-time PCR techniques.[46,47] Further studies using these more sensitive tools might help to clarify these open questions.

References

1. Shimoni A, Nagler A. Non-myeloablative stem cell transplantation (NST): chimerism testing as guidance for immune-therapeutic manipulations. Leukemia 2001;15:1967-75.
2. Dubovsky J, Daxberger H, Fritsch G, Printz D, Peters C, Matthes S, et al. Kinetics of chimerism during the early post-transplant period in pediatric patients with malignant and non-malignant hematologic disorders: implications for timely detection of engraftment, graft failure and rejection. Leukemia 1999;13:2060-9.
3. Miflin G, Stainer CJ, Carter GI, Byrne JL, Haynes AP, Russell NH. Comparative serial quantitative measurements of chimaerism following unmanipulated allogeneic transplantation of peripheral blood stem cells and bone marrow. Br J Haematol 1999;107:429-40.
4. Petit T, Raynal B, Socié G, Landman-Parker J, Bourhis J-H, Gluckman E, et al. Highly sensitive polymerase chain reaction methods show the frequent survival of residual recipient multipotent progenitors after non-T-cell-depleted bone marrow transplantation. Blood 1994;84:3575-83.
5. Molloy K, Goulden N, Lawler M, Cornish A, Oakhill A, Pamphilon D, et al. Patterns of hematopoietic chimerism following bone marrow transplantation for childhood acute lymphoblastic leukemia from volunteer unrelated donors. Blood 1996;87:3027-31.
6. Leewen van JE, van Tol MJ, Joosten AM, Wijnen JT, Verweij PJ, Khan PM, et al. Persistence of host-type hematopoiesis after allogeneic bone marrow transplantation for leukemia is significantly related to the recipient's age and/or the conditioning regimen, but is not associated with an increased risk of relapse. Blood 1994;83:3059-67.
7. Bertheas MF, Lafage M, Levy P, Blaise D, Stoppa AM, Viens P, et al. Influence of mixed chimerism on the results of allogeneic bone marrow transplantation for leukemia. Blood 1991;78:3103-6.
8. Briones J, Urbano-Ispizua A, Lawler M, Rozman C, Gardiner N, Marin P, et al. High frequency of donor chimaerism after allogeneic transplantation of CD34+ selected peripheral blood cells. Exp Hematol 1998;26:415-20.
9. Childs R, Clave E, Contentin N, Jayasekera D, Hensel N, Leitman S, 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.
10. Serrano J, Román J, Sánchez J, Jiménez A, Castillejo JA, Herrera C, et al. Molecular analysis of lineage-specific chimerism and minimal residual disease by RT-PCR of p210BCR-ABL after allogeneic bone marrow transplantation for chronic myeloid leukemia: increasing mixed myeloid chimerism and p190BCR-ABL detection precede cytogenetic relapse. Blood 2000;95:2659-65.
11. Román J, Serrano J, Jiménez A, Castillejo JA, Reina L, González MG, et al. Myeloid mixed chimerism is associated with relapse in bcr-abl positive patients after unmanipulated allogeneic bone marrow transplantation for chronic myelogenous leukemia. Haematologica 2000;85:173-80.
12. Weger de RA, Tilanus MGJ, Scheidel KC, van den Tweel J, Verdonk LF. Monitoring of residual disease and guided donor leucocyte infusion after allogeneic bone marrow transplantation by chimaerism analysis with short tandem repeats. Br J Haematol 2000;110:647-53.
13. Bader P, Hölle W, Klingebiel T, Handgretinger R, Benda N, Schlegel PG, et al. Mixed hematopoietic chimerism after allogeneic bone marrow transplantation: the impact of quantitative PCR analysis for prediction of relapse and graft rejection in children. Bone Marrow Transplant 1997;19:697-702.
14. Bader P, Beck J, Frey A, Schlegel PG, Hebarth H, Handgretinger R, et al. Serial and quantitative analysis of mixed hematopoietic chimerism by PCR in patients with acute leukemias allows the prediction of relapse after allogeneic BMT. Bone Marrow Transplant 1998;21:487-95.
15. Mackinnon S, Barnett L, Heller G, O'Reilly RJ. Minimal residual disease is more common in patients who have mixed T-cell chimerism after bone marrow transplantation for chronic myelogenous leukemia. Blood 1994;83:3409-16.
16. Wäsh R, Bertz H, Kunzmann R, Finke J. Incidence of mixed chimaerism and clinical outcome in 101 patients after myeloablative conditioning regimens and allogeneic stem cell transplantation. Br J Haematol 2000;109:743-50.
17. Wang LJ, Chou P, Gonzalez-Ryan L, Huang W, Haut PR, Kletzel M. Evaluation of mixed hematopoietic chimerism in pediatric patients with leukemia after allogeneic stem cell transplantation by quantitative PCR analysis of variable number of tandem repeat and testis determination gene. Bone Marrow Transplant 2002; 29:51-6.
18. Mattson J, Uzunel M, Tammik L, Aschan J, Ringdén O. Leukemia lineage-specific chimerism analysis is a sensitive predictor of relapse in patients with acute myeloid leukemia and myelodysplastic syndrome after allogeneic stem cell transplantation. Leukemia 2001;15:1976-85.
19. Leewen van JE, van Tol MJ, Joosten AM, Wijnen JT, Khan PM, Voosen JM. Mixed T-lymphoid chimerism after allogeneic bone marow transplantation for hematologic malignancies of children is not correlated with relapse. Blood 1993;82:1921-8.
20. Najfeld V, Burnett W, Vlachos A, Scigliano E, Isola L, Fruchtman S. Interphase FISH analysis of sex-mismatched BMT using dual color XY probes. Bone Marrow Transplant 1997;19:829-34.
21. Choi SJ, Lee KH, Lee JH, Kim S, Chung HJ, Lee JS, et al. Prognostic value of hematopoietic chimerism in patients with acute leukemia after allogeneic bone marrow transplantation: a prospective study. Bone Marrow Transplant 2000;26:327-32.
22. Schaap N, Schattenberg A, Mensink E, Preijers F, Hillegers M, Knops R, et al. Long-term follow-up of persisting mixed chimerism after partially T-cell depleted allogeneic stem cell transplantation. Leukemia 2002;16:13-21.
23. Budowle B, Chakraborty R, Giusti AM, Eisenberg AJ, Allen RC. Analysis of the VNTR locus D1S80 by the PCR followed by high-resolution PAGE. Am J Hum Genet 1991;48:137-44.
24. Stuppia L, Calabrese G, Di Bartolomeo P, Peila R, Franchi PG, Morizio E, et al. Retrospective investigation of hematopoietic chimerism after BMT by PCR amplification of hypervariable DNA regions. Cancer Genet Cytogenet 1995;85:124-8.
25. Ugozzoli L, Yam P, Petz LD, Ferrara GB, Champlin RE, Forman SJ, et al. Amplification by the polymerase chain reaction of hypervariable regions of the human genome for evaluation of chimerism after bone marrow transplantation. Blood 1991;77: 1607-15.
26. Glucksberg H, Storb R, Fefer A, Buckner CD, Neiman PE, Clif RA, et al. Clinical manifestations of graft-versus-host disease in human recipients of marrow from HLA-matched sibling donors. Transplantation 1974;18:295-304.
27. Schulman HM, Sullivan KM, Weiden PL, McDonald GB, Strike GE, Sale GE, et al. Chronic graft-versus-host syndrome in man. A long-term clinicopathological study of 20 Seattle patients. Am J Med 1980;69:204-17.
28. Horowitz MM, Gale RP, Sondel PM, Goldman JM, Kersey J, Kolb HJ, et al. Graft-versus-leukemia reactions after bone marrow transplantation. Blood 1990;75:555-62.
29. Boiron JM, Lerner D, Pigneux A, Faberes C, Bordessoule D, Turlure P, et al. Allogeneic transplantation for patients with advanced acute leukemia: a single centre retrospective study of 92 patients. Leuk Lymphoma 2001;41:285-96.
30. Bader P, Klingebiel T, Schaudt A, Theurer-Mainka U, Handgretinger R, Lang P, et al. Prevention of relapse in pediatric patients with acute leukemias and MDS after allogeneic SCT by early immunotherapy initiated on the basis of increasing mixed chimerism: a single center experience of 12 children. Leukemia 1999;13:2079-86.
31. Elmaagacli AH, Beelen DW, Becks HW, Mobascher A, Stockova J, Trzensky S, et al. Molecular studies of chimerism and minimal residual disease after allogeneic peripheral blood progenitor cell or bone marrow transplantation. Bone Marrow Transplant 1996;

18:397-403.
32. Socie G, Lawler M, Gluckman E, McCann SR, Brison O. Studies on hemopoietic chimerism following allogeneic bone marrow transplantation in the molecular biology era. Leuk Res 1995;19:497-504.
33. Bader P, Duckers G, Kreyenberg H, Hoelle W, Kerst G, Lang P, et al. Monitoring of donor cell chimerism for the detection of relapse and early immunotherapeutic intervention in acute lymphoblastic leukemias. Ann Hematol 2002;81 Suppl 2:S25-S7.
34. Ramírez M, Díaz MA, García-Sánchez F, Velasco M, Casado F, Villa M, et al. Chimerism alter allogeneic hematopoietic cell transplantation in childhood acute lymphoblastic leukemia. Bone Marrow Transplant 1996;18:1161-5.
35. González M, López-Pérez R, García-Sanz R, Pérez-Simón JA, San Miguel JF. Debate round table: comments concerning chimerism studies. Leukemia 2001;15:1986-8.
36. Bär BMAM, Schattenberg A, De Man AJM, Hoogenhout J, Boezeman J, de Witte T. Influence of the conditioning regimen on erythrocyte chimerism, graft-versus-host disease and relapse after allogeneic transplantation with lymphocyte depleted marrow. Bone Marrow Transplant 1992;27:45-52.
37. Schots R, Van Riet I, Ben Othman T, Trullemans F, De Waele M, Van Camp B. The impact of partial T cell depletion on overall transplant-related toxicity, graft function and survival after HLA-identical allogeneic bone marrow transplantation in standard risk adult patients with leukemia. Bone Marrow Transplant 2001;28: 917-22.
38. Serrano J, Román J, Herrera C, Castillejo JA, Navarro JA, Reina ML, et al. Increasing mixed haematopoietic chimerism alter BMT with total depletion of CD4+ and partial depletion of CD8+ lymphocytes is associated with a higher incidente of relapse. Bone Marrow Transplant 1999;23:475-82.
39. Roux E, Heig C, Dumont-Girard F, Chapuis B, Jeannet M, Roosnek E. Analysis of T-cell repopulation after allogeneic bone marrow transplantation: significant differences between recipients of T-cell depleted and unmanipulated grafts. Blood 1996;87:3984-92.
40. Storb R. Mixed allogeneic chimerism and graft-versus-leukemia effects in acute myeloid leukemia. Leukemia 2002;16:753-4.
41. Schaap N, Schattenberg A, Bär B, Preijers F, van de Wiel van Kemenade E, de Witte T. Induction of graft-versus-leukemia to prevent relapse after partially lymphocyte-depleted allogeneic bone marrow transplantation by pre-emptive donor leukocyte infusions. Leukemia 2001;15:1339-46.
42. Suttorp M, Schmitz N, Dreger P, Schaub J, Löffler H. Monitoring of chimerism after allogeneic bone marrow transplantation with unmanipulated marrow by use of DNA polymorphisms. Leukemia 1993;5:679-87.
43. Collins RH, Shpilberg O, Drobyski WR, Porter DL, Giralt S, Champlin R, et al. Donor leukocyte infusions in 140 patients with relapsed malignancy after allogeneic bone marrow transplantation. J Clin Oncol 1997;15:433-44.
44. Keil F, Prinz E, Kalhs P, Lechner K, Moser K, Schwarzinger I, et al. Treatment of leukemic relapse after allogeneic stem cell transplantation with cytoreductive chemotherapy and/or immunotherapy or second transplants. Leukemia 2001;15:355-61.
45. Imoto S, Murayama T, Gomyo H, Mizuno I, Sugimoto T, Nakagawa T, et al. Long-term molecular remission induced by donor lymphocyte infusions for recurrent acute myeloblastic leukemia after allogeneic bone marrow transplantation. Bone Marrow Transplant 2000;26:809-10.
46. Alizadeh M, Bernard M, Danic B, Dauriac C, Birebent B, Lapart C, et al. Quantitative assessment of hematopoietic chimerism after bone marrow transplantation by real-time quantitative polymerase chain reaction. Blood 2002;99:4618-25.
47. Maas F, Schaap N, Kolen S, Zoetbrood A, Buño I, Dolstra H, et al. Quantification of donor and recipient hemopoietic cells by real-time PCR of single nucleotide polymorphisms. Leukemia 2003;17: 621-9.

Pre-publication Report & Outcomes of Peer Review

Contributions

MB, AJ-V and JR-G designed the study, were responsible for the laboratory studies, and drafted the manuscript. MEM, JAC, MB and AJ-V were responsible for collecting the clinical data and collaborated in the statistical analysis. MB, AJ-V and JR-G analyzed and interpreted the results. AT and AH critically revised the manuscript and also gave their final approval of the version to be published. The order of the authors was established according to the level of responsibility and involvement in the research project. Primary responsibility for the paper rests with MB, AJ-V and JR-G, who contributed equally to the paper.

We would like to thank the nursing staff, the clinical staff and the residents of the Hematology units of both hospitals for taking care of our patients and facilitating the recording of data. We are grateful to Isabel Torroba for her useful technical assistance, to Ian Johnstone for help with the English style of the manuscript, and to David Prieto for his statistical assistance.

Funding

This work was supported by grants 02/1299 and 01/0662 from the Fondo de Investigaciones Sanitarias (FIS) and with funds from Cajamar-Fundación Hospital Carlos Haya. M. Barrios holds the fellowship FIS 01/F018.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Dr. Yoichi Takaue, who acted as an Associate Editor. The final decision to accept this paper for publication was taken jointly by Dr. Takaue and the Editors. Manuscript received March 27, 2003; accepted May 28, 2003.

In the following paragraphs, Dr. Takaue summarizes the peer-review process and its outcomes.

What is already known on this topic

The value of evaluating chimerism status in the prediction of leukemic relapse after allogeneic stem cell has been controversial in patients with acute leukemia. Early prediction of upcoming relapse may be of value for the early control of leukemia while it still remains as minimum residual disease.

What this study adds

This study by Barrios *et al.* examined this very important question in a large series of patients, and clearly showed the significant relationship between chimerism status and subsequent leukemic relapse/mortality.