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ORIGINAL ARTICLE

Fludarabine and cyclophosphamide based reduced intensity conditioning (RIC) regimens reduce rejection and improve outcome in Indian patients undergoing allogeneic stem cell transplantation for severe aplastic anemia

B George, V Mathews, A Viswabandya, ML Kavitha, A Srivastava and M Chandy

Department of Haematology, Christian Medical College, Vellore, Tamil Nadu, India

Thirty-five patients (25 men and 10 women) with a median age of 20 years with severe aplastic anaemia (SAA) underwent HLA identical stem cell transplantation (HSCT) using a combination of fludarabine and cyclophosphamide ± anti-thymocyte globulin between 2004 and 2006. Cyclosporine and mini methotrexate were used as GVHD prophylaxis. Graft source included peripheral blood stem cells (28) or G-CSF stimulated bone marrow (7). Two patients expired <7 days post-HSCT while 32 (91.5%) patients engrafted with a median neutrophil and platelet engraftment time of 12 days each. Three patients (8.5%) developed veno-occlusive disease while acute GVHD occurred in 29% of evaluable patients, with chronic GVHD in 32%. At a mean follow-up of 22 months, 29 (82.8%) are alive and well. When compared with 26 patients previously transplanted using Cy200/ antilymphocyte globulin, there was faster neutrophil engraftment (12 vs 16 days; $P = 0.002$) with significantly lower rejection rates (2.9 vs 30.7%; $P = 0.003$) and a superior event-free (82.8 vs 38.4%; $P = 0.001$) and overall survival (82.8 vs 46.1%; $P = 0.005$). A combination of fludarabine with cyclophosphamide ± anti-thymocyte globulin reduces rejection and improves overall and event-free survival in Indian patients undergoing HSCT for severe aplastic anaemia.

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Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is the treatment of choice for young patients with severe aplastic anaemia (SAA) who have an HLA-matched related donor, with disease-free survival rates ranging between 60 and 95% in various studies.[1–5] A number of factors have been previously identified as affecting survival after HSCT including older age, interval between diagnosis and transplantation, number of transfusions before transplant and use of cyclosporine (CsA) for graft-versus-host disease (GVHD) prophylaxis.[2,6,7] Graft rejection is an important cause of increased morbidity and mortality following HSCT for SAA, with rejection rates ranging from 10 to 30%.[8] Factors associated with high rejection rates include a long interval between diagnosis and transplant, increased number of pre-transplant blood transfusions (>20), pre-transplant infections and multiple treatments including immunosuppressive therapy before transplant.[8–11] In many developing countries, there is usually a time delay between diagnosis and HSCT due to economic and resource constraints and hence patients have multiple risk factors at the time of HSCT leading to high rejection rates and poor survival. Strategies used to reduce graft rejection include the addition of radiation therapy or anti-thymocyte globulin (ATG) to the basic conditioning regime of cyclophosphamide or the administration of donor buffy coats post transplant, but high rejection rates are still seen in developing countries with these protocols.[12] Fludarabine is a novel purine analogue that has been used for non-myeloablative transplants in older patients and has the advantage of reduced toxicity and increased immunosuppression. There have been a few reports on the use of fludarabine-based protocols in patients with aplastic anaemia including Fanconi's anaemia, resulting in reduced rejection and low toxicity.[13–15] We present our data on the use of a combination of fludarabine and cyclophosphamide, with, or without ATG, as conditioning therapy for HLA matched allogeneic stem cell transplants in aplastic anaemia.

Patients and methods

All patients who underwent HLA identical allogeneic HSCT for aplastic anaemia at the Christian Medical College Hospital between 2004 and 2006 were included in this study. All patients had a six antigen HLA matched sibling or family donor. Written informed consent was obtained from all patients before HSCT.

Correspondence: Dr B George, Department of Haematology, Christian Medical College, Vellore 632004, Tamil Nadu, India.
E-mail: biju@cmcvellore.ac.in
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High-risk patients were identified as those with any of the following risk factors –

(a) Active infection (bacterial or fungal) at the time of BMT.
(b) Failed previous immunosuppressive therapy (ALG/ATG or cyclosporine).
(c) Number of previous transfusions >20.

Transplant
All patients were nursed in HEPA-filtered rooms. The conditioning regimen used was a combination of Fludarabine and Cyclophosphamide with, or without ATG.

Graft source and engraftment
G-CSF stimulated peripheral blood stem cells was the preferred graft source. G-CSF stimulated bone marrow was used with small paediatric donors. Neutrophil engraftment was defined as the first of three consecutive days with ANC $>500/mm^3$, while platelet engraftment was defined as the first of three consecutive untransfused platelet counts $>20\,000/mm^3$. Chimerism analysis using VNTRs was done at day 30 post-HSCT. In patients who had a sex-mismatched transplant, chimerism was assessed with FISH for X and Y chromosomes. Repeat chimerism analysis was carried out after 1 month if the initial chimerism showed a mixed picture.

Antimicrobial prophylaxis

No antibacterial or antifungal prophylaxis was used. Acyclovir was started on day +1 as herpes prophylaxis and Sulfamethoxazole/Trimethoprim as prophylaxis against pneumocystis carinii was started once the WBC count was $>3\times10^9$/l with ANC $>1.5\times10^9$/l. PCR for CMV infection was checked serially starting day +30, and pre-emptive therapy with ganciclovir was instituted for CMV PCR-positive patients.

GVHD prophylaxis
GVHD prophylaxis consisted of CSA alone or in combination with short course MTX. CSA was started at a dose of 3 mg/kg/day intravenously in two divided doses, on day −1, and changed to the oral route once the counts had improved and the patient was able to take oral medication. CSA was continued at full doses for a period of 6 months and then tapered, in the absence of GVHD, over a period of 6 months to stop at 12 months following BMT. Intravenous MTX was given initially at a dose of $5\,mg/m^2$ on days +1, +3 and +6 followed by folinic acid rescue 24 h later. From October 2004, the dose of MTX was increased to $10\,mg/m^2$ on day +1 followed by $7\,mg/m^2$ on days +3 and +6. Individual doses of methotrexate were omitted in the presence of severe mucositis, bleeding or jaundice. GVHD was diagnosed on tissue biopsy – either skin, upper gastrointestinal or rectal biopsy, and treatment was initiated on clinical grounds or because of a positive biopsy, with either intravenous dexamethasone or methyl prednisolone, which was converted into oral steroids once the symptoms of GVHD had improved. If the patient was steroid refractory, second-line therapy included ATG, antilymphocyte globulin (ALG), anti-T cell antibody (OKT3), mycophenolate or daclizumab. Chronic GVHD was treated with a combination of cyclosporine and oral steroids. In patients where improvement was not satisfactory, azathioprine or thalidomide were considered.

Statistical analysis
Event-free survival (EFS) was calculated as survival in the absence of death or relapse. Overall survival (OS) included all patients who were alive on the last evaluation. For comparison of dichotomous variables, a χ^2 test was done while continuous variables were compared using either a Student's *t*-test or a Mann–Whitney *U*-test as was deemed appropriate. The probability of OS and DFS were estimated using Kaplan–Meier method. The prognostic relevance of clinical and biological variables was studied using univariate and multivariate Cox regression analysis. For all the tests, a two-sided *P*-value of 0.05 or less was considered statistically significant.

Results

There were 35 patients (25 men and 10 women) including 12 children, with a median age of 20.8 years (range: 5–43). The median time from diagnosis to HSCT was 10.4 months (range: 2–84) while the median number of transfusions before HSCT was 17 (range: 2–140). At the time of HSCT, 18 patients (51.4%) were considered as high risk (Table 1). Risk factors included active infection (bacterial or fungal) in 10, >20 transfusions in two, failed immunosuppression (ALG or CSA) in four and a second BMT in two. Two patients (6.6%) were on ventilatory support at the time of the graft infusion.

Conditioning regimens used for transplant consisted of Fludarabine $180\,mg/m^2$ over 6 days (days −7 to −2) + cyclophosphamide 120 mg/kg over 2 days (days −3 and −2) in 24 patients (Flu-Cy protocol) and fludarabine $180\,mg/m^2$ over 6 days (days −10 to −5) + cyclophosphamide 120 mg/kg over 2 days (days −6 and −5) ± ATG (ATGAM) 10 mg/kg/day for 4 days (days −4 to −1) in 11 (Flu-Cy-ATG protocol) patients.

Engraftment
The graft source was peripheral blood stem cells (PBSC) in 28 (80%) patients and G-CSF stimulated bone marrow in

Table 1 Pre-transplant characteristics of patients undergoing HSCT with a combination of Fludarabine and Cyclophosphamide ± ATG

	Flu/Cy ± ATG (n = 35)
Median age (years)	20.8 (5–43)
Sex	
Male	25
Female	10
Median time from diagnosis to BMT months	10.4 (2–84)
Median number of transfusions before BMT	17 (2–140)
High-risk patients	18 (51.4%)

seven (20%). The median cell dose was 5.3×10^8 total nucleated cells/kg for bone marrow (range: 2.1–9.9) and 6.1×10^8 MNC/kg for PBSC (range: 1.9–12.3). Thirty-two (91.5%) engrafted with two patients (5.7%) expiring <7 days post-BMT, while one patient (2.8%) had primary graft rejection. The median time to ANC >500/mm^3 was 12.3 days (range: 9–19), ANC >1000/mm^3 was 13.6 days (range: 10–19) and platelet count >20 000/mm^3 was 12.4 days (range: 8–32) (Table 2). Post-transplant chimerism data were available for 31 evaluable patients. The day 30 chimerism showed 100% donor chimerism in 30 patients (96.7%) while mixed chimerism was seen in one patient (3.3%). This patient continued to show mixed chimerism on last follow-up (97.4% donor cells) with normal blood counts.

Toxicity and GVHD

Three patients (8.5%) developed veno-occlusive disease of the liver. All responded well to supportive therapy.

Cyclosporine in combination with short course MTX was used in 31 patients (88.5%) while cyclosporine alone or combined with methylprednisolone was used as GVHD prophylaxis in four patients (13.4%). Of the 31 patients who were evaluable for GVHD, nine (29%) developed acute GVHD. Of these, seven had grade I and II GVHD while one each had grade III and grade IV GVHD. Seven patients (77.7%) responded to a combination of cyclosporine and prednisolone while two (22.3%) expired owing to GVHD or associated infection. Twenty-five patients were evaluable for chronic GVHD, and of these, eight (32%) developed chronic GVHD, which was limited in all patients. All improved with a combination of cyclosporine and prednisolone. Comparing the graft source, the incidence of acute GVHD was not significantly higher with the use of PBSC (31 vs 20%; $P=1.000$) nor was the incidence of chronic GVHD higher (30 vs 40%; $P=0.172$).

Table 2 Engraftment, toxicity and survival

	Flu/Cy±ATG (n=35)
Engraftment	32 (91.5%)
ANC>500 (days)	12.3 (9–19)
ANC>1000 (days)	13.6 (10–19)
Plt count>20 000 (days)	12.4 (8–32)
Acute GVHD	9/31 (29%)
Chronic GVHD	8/25 (32%)
Toxicity (VOD/HC)	3/35 (8.5%)
EFS	29/35 (82.8%)
Overall survival	29/35 (82.8%)

Abbreviations: EFS = event-free survival; VOD = veno-occlusive disease.

Survival

Four patients (11.4%) expired <2 weeks following HSCT, while the day −100 transplant-related mortality was 17.1%. At a median follow-up of 22 months, 29 (82.8%) are alive and well. Among the 17 patients who did not have any high-risk features at the time of HSCT, the overall survival was 100%.

We compared engraftment, toxicity and survival between patients who received ATG ($n=11$) and those who did not ($n=24$). There was no difference in the median time to neutrophil and platelet engraftment, and the incidence of acute GVHD and survival (Table 3) were also similar between both groups.

A historical comparison was carried out with 26 patients who underwent allogeneic HSCT for aplastic anaemia at our centre between 1990 and 2004 using either cyclophosphamide (200 mg/kg over 4 days) alone ($n=2$) or in combination with ALG (Lymphoglobuline, Pasteur Merieux 90 mg/kg over 3 days) ($n=24$) as the conditioning regimen. Bone marrow was used as the graft source in all patients. Table 4 shows the comparative data between groups. The number of patients in the high-risk category was similar (42% in the Cy200/ALG group; 51% in the Flu/Cy group) although the number of transfusions in the past, was significantly higher in the fludarabine group (Table 4). There was faster neutrophil (12.3 vs 16.8 days; $P=0.002$) and platelet (12.4 vs 26.2 days; $P=0.000$) engraftment in the fludarabine group, with significantly lower rejection rates (2.9 vs 30.7%; $P=0.003$) as compared to the Cy200/ALG group. There was only one primary graft failure in the fludarabine group as compared to three primary graft failures and five secondary rejections in the Cy/ALG group. OS was significantly higher in the fludarabine group (82.8 vs 46.2%; $P=0.017$) as compared to the Cy200/ALG group (Figure 1) as was the event-free survival (EFS) (82.8 vs 38.5%; $P=0.004$) (Figure 2).

Discussion

In developing countries, there is usually a delay between the diagnosis of SAA and the HSCT procedure during which patients receive multiple transfusions, develop recurrent infections or have major bleeding episodes. Many patients

Table 3 Comparison of engraftment, GVHD and survival in the Flu-Cy vs Flu-Cy-ATG group

	Flu/Cy (n=24)	*Flu/Cy/ATG* (n=11)	P-*value*
Cell dose ($\times 10^8$ TNC/kg)	6.1 (BM), 6.8[a] (PBSC)	5.7 (BM)	
ANC>500/mm^3 (days)	12±2.2	12.8±2.6	0.190
ANC>1000/mm^3 (days)	13.1±2.5	14.5±2.6	0.113
Platelet count>20 000/mm^3 (days)	11.3±5.9	14.5±5.2	0.069
Acute GVHD	4/20 (20%)	5/11 (45.4%)	0.217
OS	19/24 (79.1%)	10/11 (90.9%)	0.260

Abbreviation: TNC = total nucleated cells.
[a]Denotes mononuclear cells/kg (MNC/kg).

Table 4 Pre-transplant characteristics, engraftment, rejection, toxicity and survival between Fludarabine and Cy200/ALG group

	Fludarabine group (n = 35)	*Cy200/ALG group (n = 26)*	P-*value*
Median age (years)	20.8 ± 10.7	17.7 ± 8.3	0.293
Time interval from diagnosis to BMT	11.7 months	6.5 months	0.144
Number of transfusions before BMT	17.4	10.1	0.042
'High-risk' patients	18 (51.4%)	11 (42.3%)	0.310
ANC > 500/mm^3 (days)	12.3 ± 2.3	16.8 ± 9.3	0.002
ANC > 1000/mm^3 (days)	13.6 ± 2.6	17.9 ± 9.5	0.017
Platelet count > 20 000/mm^3 (days)	12.4 ± 5.8	26.2 ± 19.1	0.000
Acute GVHD	9/31 (29%)	6/22 (27.2%)	0.767
Rejection	1/35 (2.9%)	8/26 (30.7%)	0.003
DFS	29/35 (82.8%)	10/26 (38.5%)	0.001
OS	29/35 (82.8%)	12/26 (46.2%)	0.005

Abbreviation: DFS = disease-free survival.

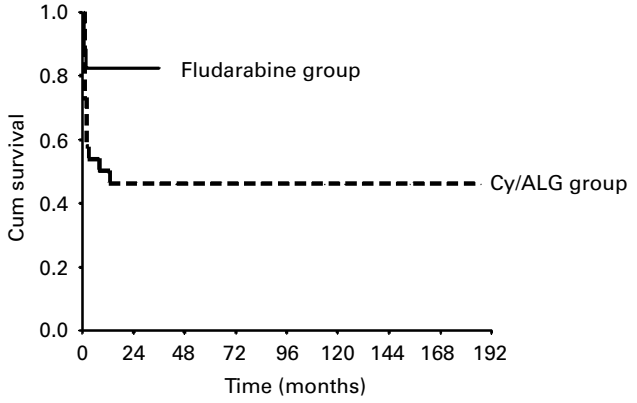

Figure 1 OS comparing Fludarabine and Cy200/ALG group.

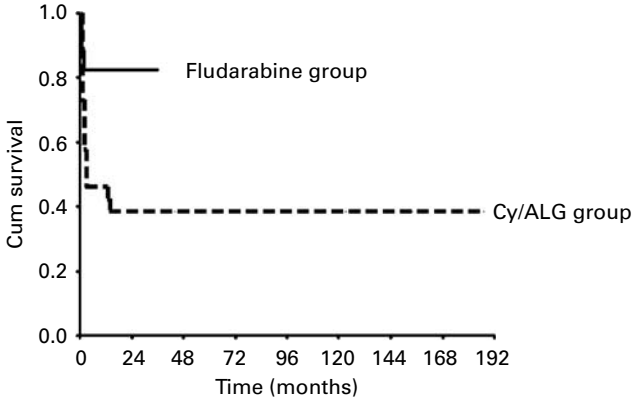

Figure 2 EFS comparing Fludarabine and Cy200/ALG group.

may also receive immunosuppressive therapy with either ALG or cyclosporine and are referred for transplant only if these fail. All of these factors can contribute to a poor outcome with HSCT, related mainly to graft rejection and infection. Fludarabine-based protocols have been used in both related and unrelated transplants for aplastic anaemia with reduced rejection rates and minimal GVHD.[14,16,17] We started using the fludarabine-based protocol reported by Slavin[18] for patients with aplastic anaemia since the standard CY200/ALG protocol was associated with high rejection rates. The busulfan in the original protocol was substituted with cyclophosphamide in 2004 since we believed that 'immunosuppression' rather than 'myeloablation' was the key component of a good conditioning regimen for aplastic anaemia. Using a combination of fludarabine, cyclophosphamide ± ATG, we have been able to reduce rejection rates to < 3% which is significantly lower than in our historical controls (30.7%) when a combination of cyclophosphamide and ATG was used for conditioning.

Gupta *et al*.[19] used a combination of cyclophosphamide and *in vivo* anti-CD52 monoclonal antibodies in heavily transfused patients with aplastic anaemia and showed adequate engraftment with a low incidence of GVHD, but graft rejection rates were still very high (24%). The low incidence of GVHD in our study is similar to data available on fludarabine based transplants in aplastic anaemia from India, Mexico and Israel,[20–23] but it is very different from NIH data[24] where the incidence of acute GVHD II–IV was 65% despite using Cyclosporine ± Methotrexate as GVHD prophylaxis and equine ATG as part of the conditioning regimen. In our series, the exclusion of ATG from the conditioning regimen did not adversely affect the incidence of rejection or GVHD (45.4% with ATG vs 20% without ATG) despite PBSC being the graft source in 80% of patients. Garza *et al*.[25] in their study on multi-transfused patients with aplastic anaemia also suggest that the use of PBSC without the use of ATG was associated with a good outcome. We preferred to use PBSC as the graft source since > 50% of patients were considered high risk (either multiple transfusions or active infection at time of HSCT) and hence a higher cell dose (including CD34 dose) should reduce the risk of rejection while a faster neutrophil engraftment would help in reducing infection related mortality. Retrospective data analysis from the IBMTR/EBMT seemed to suggest a worse outcome with the use of PBSC[26] but in our small cohort of patients, the use of PBSC was not associated with an increased risk of GVHD or inferior survival. A longer follow-up will help us in studying the long-term impact of using PBSC. Whether the presence of fludarabine in the conditioning regimen had a moderating influence on GVHD and hence on long-term survival will need to be addressed in larger studies. The long-term remission rates of > 80% in this high-risk

population make this protocol an attractive option for allogeneic HSCT, especially in developing countries. These data compare favourably with EBMT data and other single-centre studies where long-term survival is 65–75% in patients undergoing HLA identical sibling transplantation using either Cy/ATG or fludarabine-based regimens.[27–31] Interestingly, among patients who fulfilled the criteria for low-risk, survival rates were 100% suggesting that a fludarabine based protocol may be useful even in patients who are minimally transfused.

In conclusion, a combination of fludarabine with cyclophosphamide $\pm$ ATG ensures adequate engraftment with minimal toxicity and low rejection rates in patients with aplastic anaemia. The use of fludarabine and the need for ATG in standard conditioning regimens for aplastic anaemia must be further explored in larger studies.

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