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Different Outcomes between Cyclophosphamide Plus Horse or Rabbit Antithymocyte Globulin for HLA-Identical Sibling Bone Marrow Transplant in Severe Aplastic Anemia

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The standard regimen for HLA-identical sibling bone marrow transplant (BMT) in severe aplastic anemia (SAA) is cyclophosphamide (Cy) and horse antithymocyte globulin (ATG). Horse ATG has been replaced by rabbit ATG in many countries due to the unavailability of the former product. This study was designed to assess if these ATG preparations are interchangeable in the preparative regimen for matched related BMT in SAA. Forty consecutive BMTs were retrospectively analyzed: 20 received Cy plus horse ATG and 20 received Cy plus rabbit ATG as the preparative regimen. Conditioning with rabbit ATG was protective against acute graft-versus-host disease (aGVHD) grades II-IV and moderate-severe chronic GVHD (cGVHD), with incidence rates of 0% versus 35.2% ($P = .009$) and 0% versus 34.0% ($P = .04$), respectively. On day +100, the probability of proven/probable invasive fungal disease (IFD) was higher in patients conditioned with rabbit ATG, 31.2% versus 5.5%, respectively ($P = .04$). Earlier cytomegalovirus (CMV) reactivation (40 versus 50 days; $P = .02$) was observed with rabbit ATG. An inferior lymphocyte count on days +30 (0.360 versus 0.814 $\times$ 10^9/L; $P = .01$) and +90 (0.744 versus 1.330 $\times$ 10^9/L; $P = .006$) was noticed in recipients of rabbit ATG. The incidence of stable mixed chimerism was higher in recipients of rabbit ATG (18.2% versus 80%, respectively; $P = .004$). These results suggest that horse and rabbit ATG preparations have different biological and clinical properties and should not be used interchangeably in the preparative regimen for related BMT in SAA.

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INTRODUCTION

Allogeneic bone marrow transplant (BMT) remains the first-line treatment for patients with severe aplastic anemia (SAA) younger than 40 years with an HLA-identical sibling donor [1,2]. The current standard preparative regimen for matched-related BMT in SAA is high-dose cyclophosphamide (Cy) and antithymocyte globulin (ATG) [1,2]. The majority of studies that have evaluated this preparative regimen used the horse ATG preparation [3-6]. Despite the lack of studies demonstrating the interchangeability of different ATG preparations in the conditioning regimen for BMT in SAA, horse ATG has been replaced by rabbit ATG due to the unavailability of the former drug in Latin America, Europe, and Japan [1]. Regardless of some common properties, rabbit ATG has a stronger lymphocytotoxicity due to its higher affinity to human lymphocytes and its extended half-life [7,8]. Notwithstanding its higher immunosuppressive quality, rabbit ATG was inferior to horse ATG as first-line treatment for SAA, with lower rates of hematological response and survival [9,10]. In BMT, rabbit ATG has been used in the preparative regimen to reduce the incidence of graft-versus-host disease (GVHD), particularly in unrelated BMT; however, its use has been associated with an increase in infectious complications and nonrelapse mortality [11-15].

As horse and rabbit ATG preparations have different biological and clinical properties, we have

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speculated if these drugs are interchangeable in the preparative regimen for matched related BMT in SAA. Therefore, we have retrospectively compared the outcomes of patients with SAA who received either Cy plus rabbit ATG or Cy plus horse ATG as the conditioning regimen for HLA-identical sibling BMT.

PATIENTS AND METHODS

Eligibility

All patients with SAA submitted to an HLA-identical sibling BMT between June 1995 and September 2011 at the Brazilian National Cancer Institute were identified. Exclusion criteria were abnormal cytogenetics, positive diepoxybutane chromosomal breakage test, presence of paroxysmal nocturnal hemoglobinuria clone with clinical manifestations, previous invasive fungal disease (IFD), conditioning regimens without ATG, and use of peripheral blood hematopoietic stem cells. The study was approved by the local ethics committee and was in accordance with the Declaration of Helsinki.

Conditioning Regimens and GVHD Prophylaxis

Conditioning with Cy (50 mg/kg on days −5 to −2) plus horse ATG (lymphoglobulin 30 mg/kg on days −5 to −3) was performed from June 1995 to October 2004. Due to the unavailability of the horse preparation in Brazil, rabbit ATG (thymoglobulin 2 mg/kg on days −4 to −1) plus Cy at the same dose has become the current preparative regimen since November 2004.

All patients received GVHD prophylaxis with cyclosporine and short-course methotrexate, which has not changed during the study period. Cyclosporine was adjusted to target a serum level between 200 and 400 ng/mL and discontinued 12 months after BMT whenever possible.

Supportive Care and Infectious Prophylaxis

Transplantations were performed in sealed single rooms with high-efficiency particulate air filters and positive air pressure. Antiviral prophylaxis consisted of acyclovir from conditioning until 1 year posttransplantation. Weekly cytomegalovirus (CMV) pp65 antigenemia was performed from neutrophil engraftment until day +100 or beyond in patients with prolonged systemic immunosuppression. Ganciclovir was used as preemptive therapy in cases with positive antigenemia. Epstein-Barr virus (EBV) PCR was not routinely performed, except in cases with clinical suspicion of posttransplantation lymphoproliferative disorder (PTLD). Antifungal prophylaxis was started after conditioning until neutrophil engraftment or beyond in cases with extended immunosuppression, as follows: patients in the Cy plus horse ATG received fluconazole, and patients in the Cy plus rabbit ATG group received fluconazole (60%), caspofungin (25%), or voriconazole (15%). Granulocyte colony-stimulating factor was administered only to patients with neutropenia and life-threatening infections.

Endpoints and Definitions

The variables analyzed were neutrophil and platelet engraftments, acute graft-versus-host disease (aGVHD) and chronic GVHD (cGVHD), CMV reactivation, and disease until day +100, IFD until day +100, PTLD, chimerism evolution, graft rejection, and autologous recovery.

Neutrophil engraftment was defined as the first of 3 consecutive days with a neutrophil count $\geq 0.500 \times 10^9$/L, and platelet engraftment as the first of 7 consecutive days with a platelet unsupported count $\geq 20 \times 10^9$/L. Cases of aGVHD and cGVHD were diagnosed and classified according to the 1994 consensus conference and the National Institutes of Health report, respectively [16,17]. CMV reactivation was defined as the presence of one or more positive cells on CMV pp65 antigenemia assay, and CMV disease was defined as the combination of symptoms and/or signs secondary to tissue lesion with detection of the virus [18]. Diagnosis of IFD was based on the criteria by the European Organization for Research and Treatment of Cancer/Mycoses Study Group, with the exclusion of possible IFD from analysis [19]. PTLD diagnosis was based on the combination of a proper clinical picture and EBV detection on the involved tissue [20]. Graft rejection and autologous recovery were specified as previously defined [21]. Chimerism was monitored with PCR-based analysis of variable numbers of tandem repeats in 90.9% and 100% of patients in the Cy plus horse ATG and Cy plus rabbit ATG groups, respectively, and cytogenetic analysis was performed in the remaining cases. Patients were classified in 3 categories regarding chimerism evolution: full donor chimerism in cases without evidence of recipient cells any time after BMT; transient mixed chimerism in cases with mixed populations of donor and recipient cells with later conversion to full donor chimerism; and stable mixed chimerism in patients with continuous mixed chimerism.

Statistical Analysis

The chi-square or Fisher exact test were used to compare categorical variables, and the Mann-Whitney nonparametric test was used for continuous variables. Neutrophil and platelet engraftments, aGVHD and cGVHD, IFD, and CMV reactivation were analyzed as time-to-event outcomes with death by other causes treated as a competitive event. Patients who did not develop aGVHD, IFD, or CMV reactivation at day +100 were censored at that point in time.

Table 1. Comparison of Baseline Characteristics According with the Preparative Regimen

	Cy Plus Horse ATG (N = 20)	Cy Plus Rabbit ATG (N = 20)	*P* Value
Age (years), median (range)	20 (4-48)	21.5 (7-57)	.90
Male gender	12 (60%)	11 (55%)	.75
VSAA at diagnosis	6 (30%)	4 (20%)	.46
Previous ATG	2 (10%)	5 (25%)	.40
BMT as first-line treatment	6 (30%)	7 (35%)	.74
Interval diagnosis-BMT (days), median (range)	170.5 (49-2659)	149 (56-1106)	.70
Pre-BMT hematologic parameters			
ANC ($\times 10^9$/L), median (range)	0.392 (0.068-2.623)	0.510 (0.021-1.544)	.31
ALC ($\times 10^9$/L), median (range)	1.387 (0.170-8.356)	1.764 (0.455-6.013)	.16
Less than 20 units of RBC before BMT	8 (40%)	12 (60%)	.20
Serum ferritin before BMT (mg/dL), median (range)	1562 (420-7564)	1215 (187-3485)	.22
Female donor	9 (45%)	7 (35%)	.51
Gender mismatch	7 (35%)	10 (50%)	.33
ABO match	12 (60%)	11 (55%)	.75
CMV seropositive donor	13 (65%)	14 (70%)	.74
Nucleated cell dose ($\times 10^8$/Kg), median (range)	3.40 (1.16-5.40)	2.49 (1.01-5.15)	.02
G-CSF before day +15	4 (20%)	7 (35%)	.28

Cy indicates cyclophosphamide; ATG, antithymocyte globulin; VSAA, very severe aplastic anemia; BMT, bone marrow transplantation; ANC, absolute neutrophil count; ALC, absolute lymphocyte count; CMV, cytomegalovirus; G-CSF, granulocyte colony-stimulating factor.

The proportional hazards model of Fine and Gray was used to compare cumulative incidence rates between the groups, based on the tutorial by Scrucca et al. [22]. Overall survival was measured from the date of transplantation to death or last follow-up and analyzed with the Kaplan-Meier method with comparisons using the log-rank test. Patients submitted to a second BMT were censored at the time of the second BMT, except for survival analysis. All *P* values were 2-sided, with $P < .05$ indicating statistical significance. Registration and analysis of data were conducted using IBM SPSS version 15 software (IBM, Armonk, NY). Cumulative incidence and the Fine and Gray test were estimated using R-project version 2.13.1, the R Project for Statistical Computing, 2011 (http://www.r-project.org/).

RESULTS

Comparison of Baseline Characteristics According to the Preparative Regimen

Among the 40 patients with SAA who were eligible for this study, 20 received Cy plus horse ATG and 20 received Cy plus rabbit ATG as the preparative regimen. As shown in Table 1, except for a difference in the nucleated cell dose, all other baseline parameters were comparable between the groups.

Graft-versus-Host Disease

The day +100 probability of aGVHD grades II-IV was 35.2% (95% confidence interval [CI], 13.8%-57.7%) and 0% (95% CI, not applicable) in patients receiving horse ATG and rabbit ATG, respectively ($P = .009$) (Figure 1A).

The 3-year cumulative incidence of moderate-severe cGVHD was 34% (95% CI, 11.5%-58.2%) and 0% (95% CI, not applicable) in recipients of horse and rabbit ATG, respectively ($P = .04$) (Figure 1B). As expected, patients with previous aGVHD grades II-IV were more likely to develop moderate-severe cGVHD (odds ratio, 66.0; 95% CI, 4.8-902.1; $P = .02$).

Infectious Complications

The day + 100 probability of proven/probable IFD was 5.5% (95% CI, 0.3%-23.0%) in recipients of horse ATG and 31.2% (95% CI, 12.1%-52.7%) in recipients of rabbit ATG ($P = .04$) (Figure 1C). The IFD diagnosed in patients conditioned with rabbit ATG were invasive aspergillosis (n = 2), disseminated fusariosis (n = 2), and invasive candidiasis (n = 2), with a median time from BMT to diagnosis of IFD of 8 days (range, −3 to 29 days). Only 1 case of IFD was observed in the Cy plus horse ATG group, in a patient who developed disseminated fusariosis on day +14.

The day +100 cumulative incidence of CMV reactivation was similar between patients conditioned with horse and rabbit ATG, 76.4% (95% CI, 46.0%-91.1%) and 76.4% (95% CI, 46.0%-91.1%), respectively ($P = .36$) (Figure 1D). However, the median time from BMT to CMV reactivation was shorter in recipients of rabbit ATG (40 versus 50 days; $P = .02$). Only 1 case of CMV disease was observed in a patient conditioned with Cy plus rabbit ATG who later developed pneumonitis.

Hematopoietic Recovery, Chimerism Evolution, and Rejection

No statistically significant difference in the CI of neutrophil engraftment on day +28 was observed between the groups: 100% (95% CI, not applicable) with Cy plus horse ATG and 93.8% (95% CI, 52.5%-99.3%) with Cy plus rabbit ATG ($P = .78$). The same is true for platelet engraftment on day +28: 82.1% (95% CI, 51.0%-94.4%) in recipients of horse

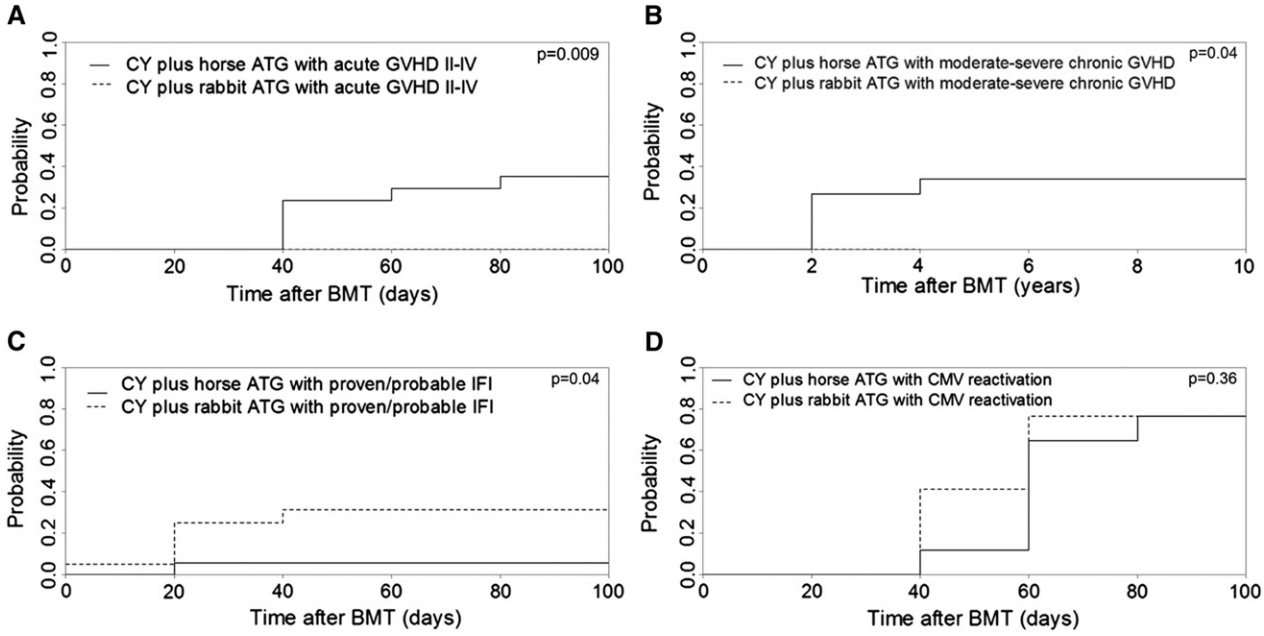

Figure 1. Cumulative incidence curves according to the preparative regimen: cyclophosphamide (Cy) plus horse antithymocyte globulin (ATG) and Cy plus rabbit ATG. Day +100 probability of grades II-IV acute graft-versus-host disease (aGVHD) (A); cumulative incidence of moderate-severe chronic GVHD (cGVHD) after bone marrow transplantation (BMT) (B); day +100 probability of proven/probable invasive fungal infection (C); and day +100 probability of cytomegalovirus (CMV) reactivation (D).

ATG and 51.8% (95% CI, 25.6%-72.8%) in recipients of rabbit ATG (P = .28). By contrast, the median absolute lymphocyte count (ALC) was lower in patients conditioned with rabbit ATG in comparison with horse ATG on days + 30 (0.360 versus 0.814 × 10^9/L; P = .01, respectively) and +90 (0.744 versus 1.330 × 10^9/L; P = .006, respectively). This difference was not statistically significant on days +60, +120, and +180 (Figure 2A).

Chimerism assessment was available in 26 of the 30 patients who survived beyond day +100 (86.7%). The distributions of full donor, transient mixed, and stable mixed chimerism were different between patients conditioned with horse ATG (81.8%, 0%, and 18.2%, respectively) and rabbit ATG (13.3%, 6.7%, and 80%, respectively) (P = .002). A trend toward an inferior median ALC was observed in patients with mixed chimerism in comparison with those with full donor chimerism on days + 30 (0.478 versus 0.770 × 10^9/L; P = .07), +60 (0.745 versus 1.031 × 10^9/L; P = .03), and +90 (0.809 versus 1.270 × 10^9/L; P = .07) (Figure 2B). Only 2 cases of graft rejection were noticed: 1 primary graft rejection in the Cy plus rabbit ATG group and 1 secondary graft rejection in the Cy plus horse ATG group. No case of autologous recovery was observed. The 2-year probability of interruption of calcineurin inhibitor (CNI) was higher in recipients of horse ATG: 85.2% (95% CI, 45.3%-96.8%) versus 48.1% (95% CI, 11.7%-77.9%; P = .001). The median time to interruption of CNI was longer in patients conditioned with rabbit ATG (574 versus 372 days; P = .004).

Survival and Mortality

The median follow-up was different between the 2 preparative regimens: 1660 days (range, 7-5139 days) for the Cy plus horse ATG group and 271 days (range, 13-1762 days) for the Cy plus rabbit ATG group (P = .04). The day +100 mortality rate was similar between recipients of horse and rabbit ATG: 25% (95% CI, 8.7%-45.4%) and 25% (95% CI, 8.7%-45.4%), respectively (P = .87). Likewise, the 1-year survival was similar between the 2 regimens: 65% in the Cy plus horse ATG group and 63.3% in the Cy plus rabbit ATG group (P = .87).

The main cause of death was GVHD-related complications in recipients of horse ATG (4 of 10: 2 with aGVHD and 2 with cGVHD) and infectious-related in recipients of rabbit ATG (4 of 8: invasive fusariosis in 2 patients, aspergillosis in 1, and respiratory syncytial virus pneumonia in 1). Only 1 case of PTLD was observed in a patient conditioned with Cy plus horse ATG.

DISCUSSION

Our study demonstrates that horse and rabbit ATG are not interchangeable when used with Cy in the conditioning regimen for SAA. We observed that the use of horse ATG was associated with a more efficient donor lymphocyte engraftment, as shown by the higher ALC at different time points and the higher proportion of patients with full donor chimerism. Consequently, this was translated into higher rates of

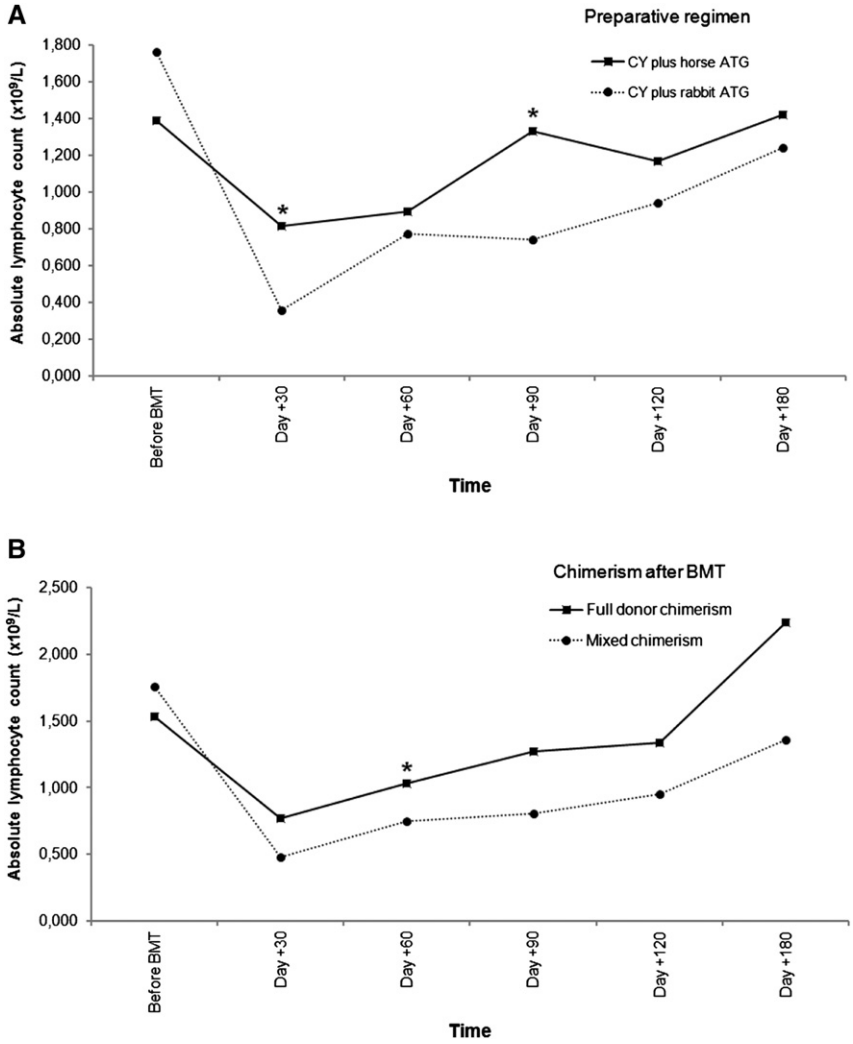

Figure 2. Evolution of the median absolute lymphocyte count (ALC). Evolution of the median ALC according to the preparative regimen: cyclophosphamide (Cy) plus horse antithymocyte globulin (ATG) and Cy plus rabbit ATG (A); and evolution of the median ALC in patients with full donor and mixed chimerism (B). Asterisks denote significant differences between the groups ($P < .05$ on the basis of the Mann-Whitney nonparametric test).

aGVHD and cGVHD, with 4 deaths related to this complication. On the other hand, the use of rabbit ATG was associated with higher rates of infectious complications, with 3 deaths related to this complication. The net result was that the 1-year probability of survival was similar between the 2 groups.

The observed high rates of GVHD in our patients conditioned with Cy plus horse ATG were similar to previous studies [4-6], as was the protective effect of rabbit ATG against GVHD [11-13]. However, to our knowledge, our study is the first to report the distinct effects of horse and rabbit ATG preparations on the incidence of GVHD after matched related BMT for SAA.

Another remarkable finding of our study was the high rate of infectious complications, notably IFD, in patients conditioned with Cy plus rabbit ATG. The day +100 CI of IFD was higher in recipients of rabbit ATG, CMV reactivation occurred earlier, and infection-related mortality was the main cause of death in these patients. All IFD in the group conditioned with rabbit ATG occurred before day +30, a period in which neutropenia is the major risk factor for IFD [23]. The 28-day probability of neutrophil engraftment was similar between the 2 groups. By contrast, the median ALC at day +30 was significantly lower in the rabbit ATG group. Lymphopenia was identified as an independent risk factor for early IFD (ie, before day +40, in allogeneic BMT) [24]. Therefore, the high incidence of infectious complications after rabbit ATG conditioning may be related to the slow recovery of CD8+ lymphocytes, which is not observed after horse ATG conditioning [25]. Alterations in other lymphocyte subtypes may also be related to the higher infectious complications observed after conditioning with rabbit ATG.

In the present study, we observed a high incidence of mixed chimerism after conditioning with Cy plus rabbit ATG, which is probably related to the reduced-intensity nature of the SAA preparative regimens based

on Cy plus ATG and the marked T cell depletion of the graft promoted by rabbit ATG. This hypothesis is further supported by the lower median ALC in our patients with mixed chimerism compared with those with full donor chimerism. The high incidence of mixed chimerism after rabbit ATG conditioning promoted the extended use of CNI, because of the concerns related to the increased risk of late graft failure after the withdrawal of immunosuppression [21,26]. The small proportion of patients with late graft failure observed hampered any analysis of the association between mixed chimerism and late graft failure. However, an increase in the occurrence of late graft failure is anticipated with the extended follow-up and the withdrawal of CNI in recipients of rabbit ATG.

Our study suffers from various limitations, the most important being the small sample size and the retrospective nature of the study. The small sample size limited the analysis by increasing the possibility of beta error and also hampered multivariate analysis. Another limitation is that the 2 groups were not contemporary. Different standards in terms of supportive care may have occurred. In addition, since the incidence of IFD, especially those caused by molds, may be influenced by environmental factors, with different incidence rates in different periods, we cannot exclude the possibility that the higher incidence of IFD in the rabbit ATG group could be because of this fact.

Despite these limitations, the different outcomes we observed in the 2 groups are significant. Furthermore, the probability of running a prospective trial comparing these ATG preparations for related BMT in SAA is very low, because of the slow accrual of SAA patients for matched BMT and the unavailability of horse ATG in the majority of the countries.

As pointed out, the protective effect of rabbit ATG against GVHD was counterbalanced by the high incidence of infectious complications. Therefore, approaches to reduce the rate of complications related to conditioning with rabbit ATG should be pursued. Potential areas to be explored include the use of antimold prophylaxis or active monitoring with biomarkers (such as serum galactomannan) in periods at greater risk, as well as the administration of smaller doses of rabbit ATG, as reported in other settings [13-15]. In addition, the administration of rabbit ATG apart from bone marrow infusion could partially reduce the T cell depletion of the graft and, accordingly, the deleterious effects of rabbit ATG on infectious complications and on the acquisition of full donor chimerism. Finally, thymoglobulin, the rabbit ATG preparation used in our patients, seems to be more immunosuppressive than ATG-Fresenius, and this may also affect the outcomes [27].

In summary, our results suggest that horse and rabbit ATG preparations have different biological and clinical properties, as already demonstrated for first-line immunosuppression in SAA [9,10]. These ATG preparations should not be used interchangeably in the preparative regimen of matched sibling BMT in SAA, and rabbit ATG is associated with lower rates of aGVHD and cGVHD but higher rates of infectious complications and mixed chimerism in comparison with horse ATG.

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