ORIGINAL ARTICLE

Horse versus Rabbit Antithymocyte Globulin in Acquired Aplastic Anemia

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ABSTRACT

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BACKGROUND

In severe acquired aplastic anemia, hematopoietic failure is the result of immune-mediated destruction of bone marrow stem and progenitor cells. Immunosuppressive therapy with antithymocyte globulin (ATG) plus cyclosporine is an effective alternative to stem-cell transplantation and improves blood counts and survival. Although horse ATG is the standard therapy, rabbit ATG is more potent in depleting peripheral-blood lymphocytes and is preferred in other clinical circumstances.

METHODS

From December 2005 through July 2010, we performed a randomized trial comparing these two ATG formulations in conventional regimens. Patients were treated at a single facility. The primary outcome was hematologic response at 6 months, as determined by blood counts. The study was designed to enroll 60 patients each for the rabbit-ATG and horse-ATG groups and was powered to detect a difference of 25 percentage points in the response rate.

RESULTS

A large, unexpected difference was observed in the rate of hematologic response at 6 months in favor of horse ATG (68%; 95% confidence interval [CI], 56 to 80) as compared with rabbit ATG (37%; 95% CI, 24 to 49; P<0.001). Overall survival at 3 years also differed, with a survival rate of 96% (95% CI, 90 to 100) in the horse-ATG group as compared with 76% (95% CI, 61 to 95) in the rabbit-ATG group (P=0.04) when data were censored at the time of stem-cell transplantation, and 94% (95% CI, 88 to 100) as compared with 70% (95% CI, 56 to 86; P=0.008) in the respective groups when stem-cell–transplantation events were not censored.

CONCLUSIONS

In a randomized study, rabbit ATG was inferior to horse ATG as a first treatment for severe aplastic anemia, as indicated by hematologic response and survival. (Funded by the Intramural Research Program of the National Institutes of Health; ClinicalTrials.gov number, NCT00260689.)

ACQUIRED APLASTIC ANEMIA IN ITS SEvere form is fatal without treatment. The disease is characterized pathologically by an "empty" bone marrow, in which hematopoietic precursor cells are replaced by fat, resulting in pancytopenia.[1] Severe aplastic anemia was first definitively treated with the development of stem-cell transplantation in the 1970s. The serendipitous observation of autologous marrow reconstitution in a few patients with rejected grafts suggested that the conditioning agents required for transplantation might themselves be therapeutic.[2] Purposeful immunosuppression induced by the infusion of antithymocyte globulin (ATG), polyclonal antibodies generated in animals by inoculation with human thymocytes, proved to be effective, with long-term survival that was similar to the results of stem-cell transplantation from a histocompatible sibling.[3,4] An immune mechanism of hematopoietic cell destruction was inferred from the success of ATG, and subsequent research in the laboratory and in animal models confirmed that progenitor and stem cells were targeted by immune effector cells and cytokines.[1] Cyclosporine added to ATG improved the response rate and survival, as compared with ATG alone,[5] a finding that is consistent with the pathophysiological features of the disorder.

In the 1980s and 1990s, most formal studies of the efficacy of ATG therapy in patients with severe aplastic anemia that were conducted in Europe, Japan, and the United States used horse ATG, with hematologic responses observed in about two thirds of cases.[6-9] An ATG made in rabbits has been available in the United States since 1999, when it was approved for the treatment of acute renal-allograft rejection. In our experience, horse ATG as a first therapy for severe aplastic anemia has yielded a hematologic response rate of 60 to 65%,[10-12] and we wished to improve this rate.

We hypothesized that rabbit ATG would result in a higher response rate than horse ATG as a first treatment, for several reasons. First, in comparison trials, rabbit ATG was superior to horse ATG in preventing and reversing acute renal-allograft rejection.[13,14] Second, in patients with severe aplastic anemia, rabbit ATG has been effective as salvage therapy for refractory disease or relapse after initial therapy with horse ATG.[15,16] Third, in comparison with horse ATG, rabbit ATG more efficiently depletes lymphocytes in vivo and is more cytotoxic on a weight basis in vitro.[17]

Fourth, and possibly relevant to its mechanism of activity, rabbit ATG but not horse ATG induces the development of regulatory T cells from normal T cells in tissue culture, which should be beneficial in suppressing a harmful immune response.[18,19]

On the basis of these observations, rabbit ATG has been administered as a first therapy for severe aplastic anemia. However, its effectiveness for this condition has not been prospectively tested. We report on a randomized trial comparing horse ATG with rabbit ATG in patients with severe aplastic anemia who have not previously received treatment.

METHODS

STUDY PATIENTS AND OVERSIGHT

Consecutive patients older than 2 years of age who had severe aplastic anemia were enrolled from December 2005 through July 2010 at the Mark O. Hatfield Clinical Research Center of the National Institutes of Health, in Bethesda, Maryland. Patients (or their legal guardians) provided written informed consent according to a protocol approved by the institutional review board at the National Heart, Lung, and Blood Institute and available with the full text of this article at NEJM.org. The study was monitored by an external data and safety monitoring board (for details, see the Methods section in the Supplementary Appendix, available at NEJM.org). This government-sponsored study had no commercial support.

STUDY DESIGN

This original study design is shown in Figure 1 in the Supplementary Appendix. Assignment of treatment was performed with the use of a block randomization scheme in a 1:1 ratio, with the assignment probability remaining fixed over the course of the trial. The randomization schedule was based on a table of random numbers developed by the pharmacy department at the clinical center.

The study's primary end point was hematologic response at 6 months, defined as no longer meeting the criteria for severe aplastic anemia; this end point strongly correlates with transfusion independence and long-term survival.[6,12] Secondary end points included robustness of hematologic recovery, relapse, response rate at 3 months and yearly, clonal evolution, and overall survival.

Table 1. Characteristics of the Patients.*

Characteristic	Horse ATG (N = 60)	Rabbit ATG (N = 60)	P Value
Age			
Mean — yr	37.4±2.7	31.2±2.6	0.09
<18 yr — no. (%)	12 (20)	18 (30)	0.20
Male sex — no. (%)	34 (57)	37 (62)	0.58
Cause of disease — no. (%)			
Idiopathic	58 (97)	55 (92)	0.24
Hepatitis	2 (3)	5 (8)	0.24
Cell counts			
Absolute reticulocyte count/mm³	22,100±2,584	18,072±2,283	0.24
Absolute lymphocyte count/mm³	1,291±71	1,220±79	0.50
Absolute neutrophil count			
Mean/mm³	408±50	356±46	0.44
<200/mm³ — no. (%)	23 (38)	26 (43)	0.58
Platelet count/mm³	16,317±4,689	12,650±1,138	0.45
PNH clone — no. (%)			
<1%	35 (58)	41 (68)	0.25
≥1%	25 (42)	19 (32)	0.25

* Plus–minus values are means ±SD. ATG denotes antithymocyte globulin, and PNH paroxysmal nocturnal hemoglobinuria.

Relapse was defined as any requirement for further immunosuppression (cyclosporine or another course of ATG) because of decreased blood counts.[12] Clonal evolution was defined as a new clonal cytogenetic abnormality or characteristic dysplastic or leukemic changes in the bone marrow.

IMMUNOSUPPRESSIVE REGIMENS

Horse ATG (ATGAM, Pfizer) was administered at a dose of 40 mg per kilogram of body weight per day for 4 days and rabbit ATG (Thymoglobulin, Genzyme) at a dose of 3.5 mg per kilogram per day for 5 days, as previously described.[12,16] Cyclosporine was administered at a dose of 10 mg per kilogram per day (15 mg per kilogram per day for children under 12 years of age), given in divided doses every 12 hours from day 1 and continued for at least 6 months in both the horse-ATG and rabbit-ATG groups, with the dose adjusted to maintain trough blood levels of 200 to 400 ng per milliliter (for details, see the Methods section in the Supplementary Appendix).

STATISTICAL ANALYSIS

The sample size was calculated on the basis of a 6-month response rate (primary end point) of 60%

with standard horse ATG plus cyclosporine (control group).[12] On the basis of a group sequential trial design, with a two-sided test at a 5% significance level, 80% power, and one interim analysis (to be performed when data for half the total estimated number of enrolled participants per group were available for evaluation of the primary end point), we calculated that enrollment of 60 patients per group would be required to detect a difference of 25 percentage points between groups in the 6-month response rate (for details, see the Methods section in the Supplementary Appendix).

RESULTS

CHARACTERISTICS OF THE PATIENTS

A total of 120 consecutive patients, 2 to 77 years of age, were randomly assigned to horse ATG or rabbit ATG (60 in each group) (Fig. 2 in the Supplementary Appendix). Characteristics of the patients are shown in Table 1; there were no significant differences in demographic or clinical characteristics between the groups. The median follow-up was 839 days (28 months) (range, 2 to 1852 days) for all patients and 891 days (30 months) (range, 185 to 1852 days) for surviving patients.

HEMATOLOGIC RESPONSE AND RELAPSE

The hematologic response rate at 6 months was 68% (95% confidence interval [CI], 56 to 80) with horse ATG and 37% (95% CI, 24 to 49) with rabbit ATG (P<0.001) (Table 2). The response rates at 3 and 6 months with horse ATG plus cyclosporine observed in this study were in accord with our experience with this regimen at our institution.[6,10-12] The majority of patients had a response within 3 months, and only four patients in the horse-ATG group and two in the rabbit-ATG group had a response between 3 and 6 months. Among patients with a response, the increments in blood counts were similar between the two groups (Fig. 1). When only patients who could be evaluated at 6 months were analyzed, the response rate with horse ATG was 71% (95% CI, 59 to 83) as compared with 43% (95% CI, 29 to 57) with rabbit ATG (P=0.003). The subsequent treatment of patients who did not have a response at 6 months is shown according to treatment group in Figure 3 in the Supplementary Appendix.

The cumulative incidence of relapse at 3 years (calculated with the use of Kaplan–Meier estimates) did not differ significantly between the two groups: 28% (95% CI, 9 to 43) with horse ATG and 11% (95% CI, 0 to 25) with rabbit ATG (P=0.35); the relatively small number of patients with a response at 6 months, especially in the rabbit-ATG group, resulted in wide confidence intervals and loss of statistical power (Fig. 4A in the Supplementary Appendix). All patients who had a relapse (nine in the horse-ATG group and two in the rabbit-ATG group) received additional immunosuppressive therapy.

CLONAL EVOLUTION AND SURVIVAL

The cumulative incidence of clonal evolution at 3 years (in all patients, those with and those without a response) was 21% (95% CI, 7 to 33) in the horse-ATG group and 14% (95% CI, 1 to 25) in the rabbit-ATG group (P=0.69) (Fig. 4B in the Supplementary Appendix). Among patients treated with horse ATG, one each had deletion 3, deletion 5q, deletion 13q, deletion 20q, and leukemia, and four had monosomy 7. In two patients, monosomy 7 was preceded by t(12;13) and deletion 13q. In the rabbit-ATG group, five patients had monosomy 7, and one had deletion 13q.

The overall survival rate at 3 years differed significantly between the two regimens: 96% (95% CI, 90 to 100) in the horse-ATG group as compared with 76% (95% CI, 61 to 95) in the rabbit-ATG

Table 2. Hematologic Response at 3 and 6 Months to Horse ATG and Rabbit ATG.

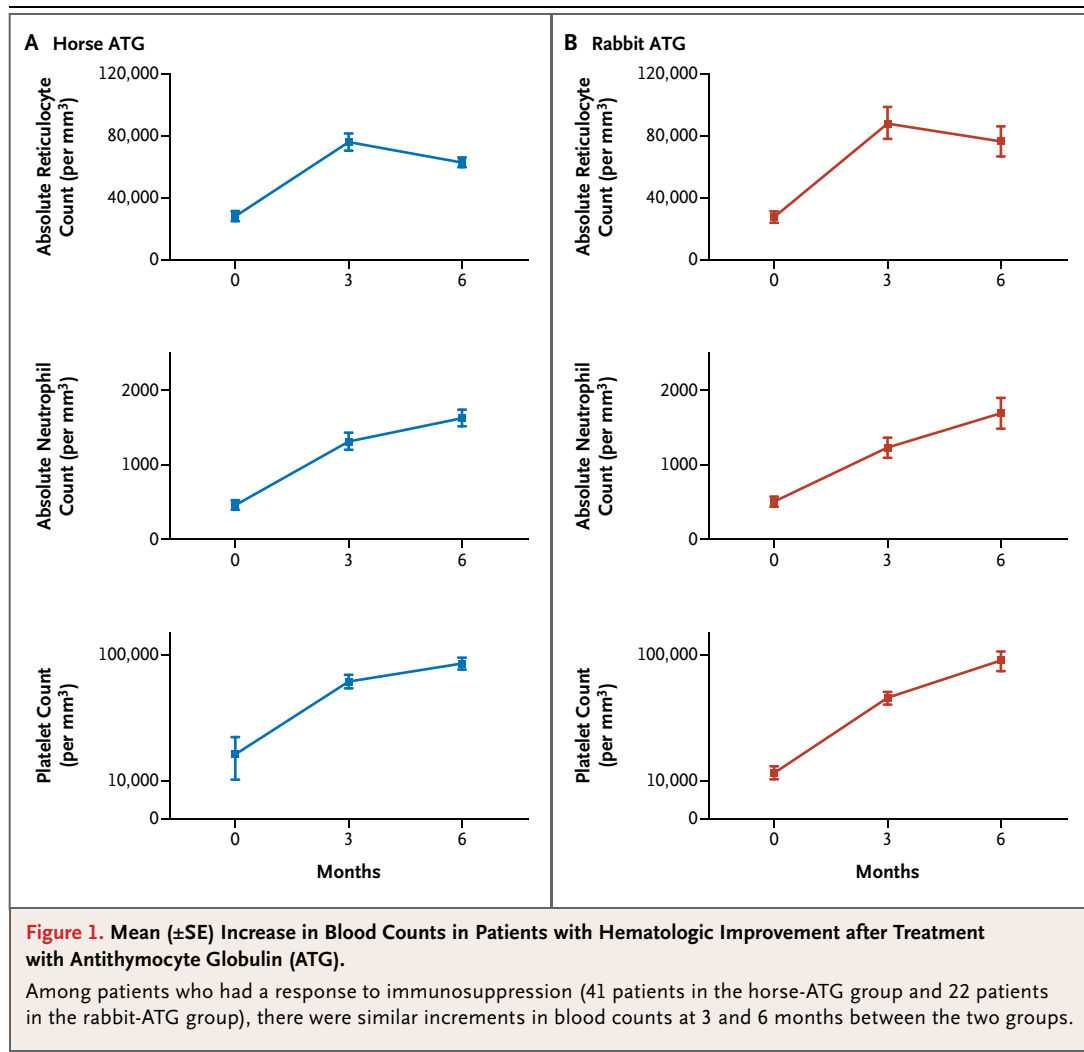| Response	Horse ATG (N=60)	95% CI	Rabbit ATG (N=60)	95% CI	P Value
	no. (%)		no. (%)		
At 3 mo	37 (62)	49–74	20 (33)	21–46	0.002
At 6 mo	41 (68)	56–80	22 (37)	24–49	<0.001

group (P=0.04), when data were censored at the time of stem-cell transplantation (Fig. 2A), and 94% (95% CI, 88 to 100) and 70% (95% CI, 56 to 86; P=0.008) in the two groups, respectively, when data were not censored at the time of stem-cell transplantation (Fig. 2B). Of the 4 deaths in the horse-ATG group, 1 each resulted from intracranial hemorrhage, sepsis, and lung cancer, and 1 occurred after stem-cell transplantation (from a sibling donor). Of the 14 deaths in the rabbit-ATG group, 2 resulted from intracranial hemorrhage and 3 from infection (1 from pneumonia, 1 from septicemia, and 1 from necrotizing fasciitis), 6 occurred after stem-cell transplantation (3 from a sibling donor and 3 from an unrelated donor), 1 was due to a traffic accident, and 2 were from unknown causes.

IN VIVO ALTERATIONS IN LYMPHOCYTE NUMBERS AND SUBPOPULATIONS

ATGs, which are polyclonal in serum, contain antibodies that recognize various antigens on cell-surface membranes, and they are cytotoxic to lymphocytes in vitro. Rapid depletion of lymphocytes occurred with both agents, but lymphopenia was more protracted after treatment with rabbit ATG, a finding that is consistent with previous reports (Fig. 3A).[17] There were strikingly lower levels of CD4+ T cells for virtually the entire 6 months before assessment of the primary end point in patients treated with rabbit ATG than in patients who received horse ATG (Fig. 3C and 3D). The numbers of regulatory T cells (defined as CD4+CD25+CD127− for this analysis)[20] were much lower in the weeks after treatment with rabbit ATG, as would be expected, given the markedly lower levels of CD4+ T cells in this group (Fig. 3E and 3F). Smaller differences were noted in the kinetics of CD8+ T cell depletion and reconstitution (Fig. 3G and 3H).

ADVERSE EVENTS

Serious adverse events are summarized in Table 1 in the Supplementary Appendix. As expected in

A   Horse ATG

B   Rabbit ATG

Figure 1. Mean (±SE) Increase in Blood Counts in Patients with Hematologic Improvement after Treatment with Antithymocyte Globulin (ATG).

Among patients who had a response to immunosuppression (41 patients in the horse-ATG group and 22 patients in the rabbit-ATG group), there were similar increments in blood counts at 3 and 6 months between the two groups.

this population, the most prevalent complications were infectious. Two patients in the horse-ATG group and nine in the rabbit-ATG group could not be evaluated at 6 months because of death or progressive disease (Fig. 2 in the Supplementary Appendix).

DISCUSSION

Immunosuppression with ATG and cyclosporine is often the first therapy administered for severe aplastic anemia, since most patients lack a histocompatible sibling donor or are not suitable candidates for stem-cell transplantation because of age, coexisting conditions, or lack of access to this treatment modality. Most published experience has involved the horse formulation of the polyclonal antibody. In the past decade, rabbit ATG plus cy-

closporine has gained in popularity because of its activity in relapsed and refractory severe aplastic anemia. In some centers in the United States, rabbit ATG has been used as the first therapy, and in Europe, Japan, and Latin America, rabbit ATG is the only formulation currently available.

The reported experience with rabbit ATG plus cyclosporine as initial therapy for severe aplastic anemia is limited to retrospective studies, with conflicting results. In a phase 2 study in the United States involving 13 patients with severe aplastic anemia, a response to rabbit ATG was observed in 12 patients (92%) at about 3 months after therapy.[21] In contrast, a retrospective analysis conducted in Brazil involving 71 patients showed a higher response rate at 6 months among those who had received horse ATG (60%) than among those treated with rabbit ATG (35%), with a sur-
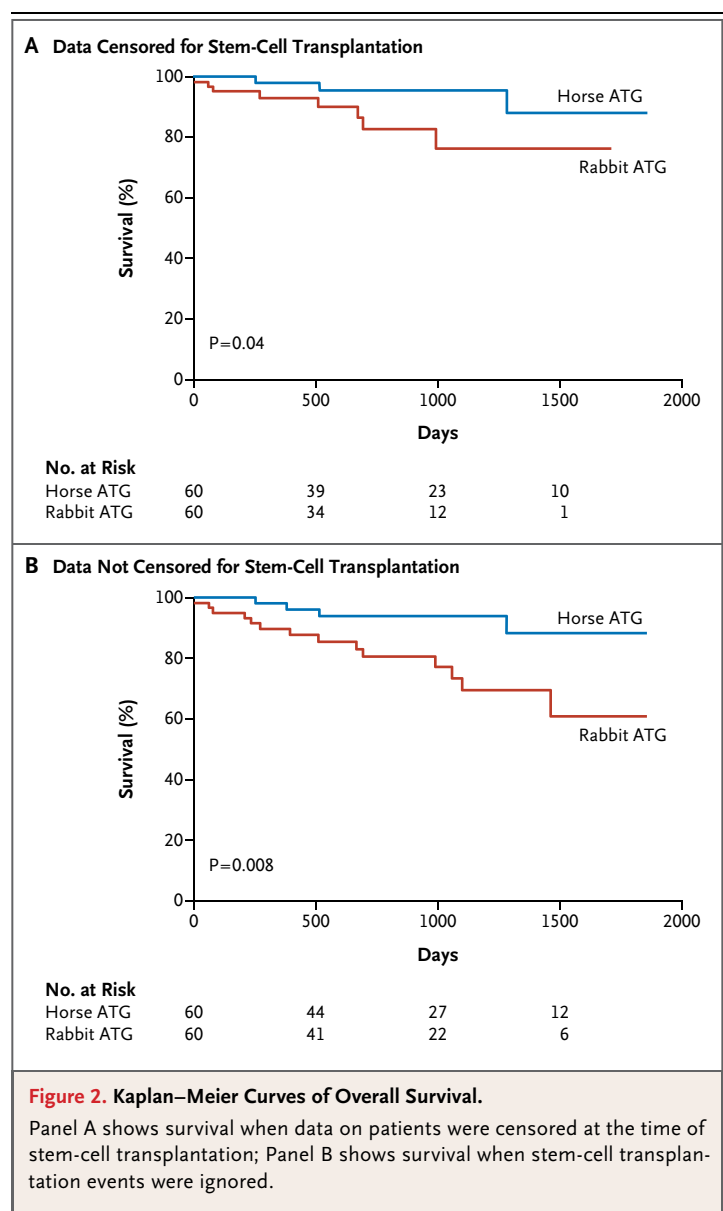
vival benefit noted in the patients receiving horse ATG. In addition, use of rabbit ATG was an independent predictor of death in a multivariate analysis in this study.[22] In a recent retrospective study from Europe, no significant difference was seen in the overall response rate between horse ATG (49%) and rabbit ATG (45%) when administered as a first-line therapy,[23] but the response rate of 49% with horse ATG was markedly lower than reported response rates of 60 to 70% with this agent in large prospective studies in the United States, Europe, and Japan.[1]

In our randomized, prospective trial, rabbit ATG plus cyclosporine was inferior to horse ATG plus cyclosporine when administered as a first-line treatment. The hematologic response rate with rabbit ATG was about half that with horse ATG, which translated into about a 25% lower survival rate at 3 years. Despite a relatively short period of follow-up, the rates of relapse and clonal evolution did not differ significantly between the two groups.
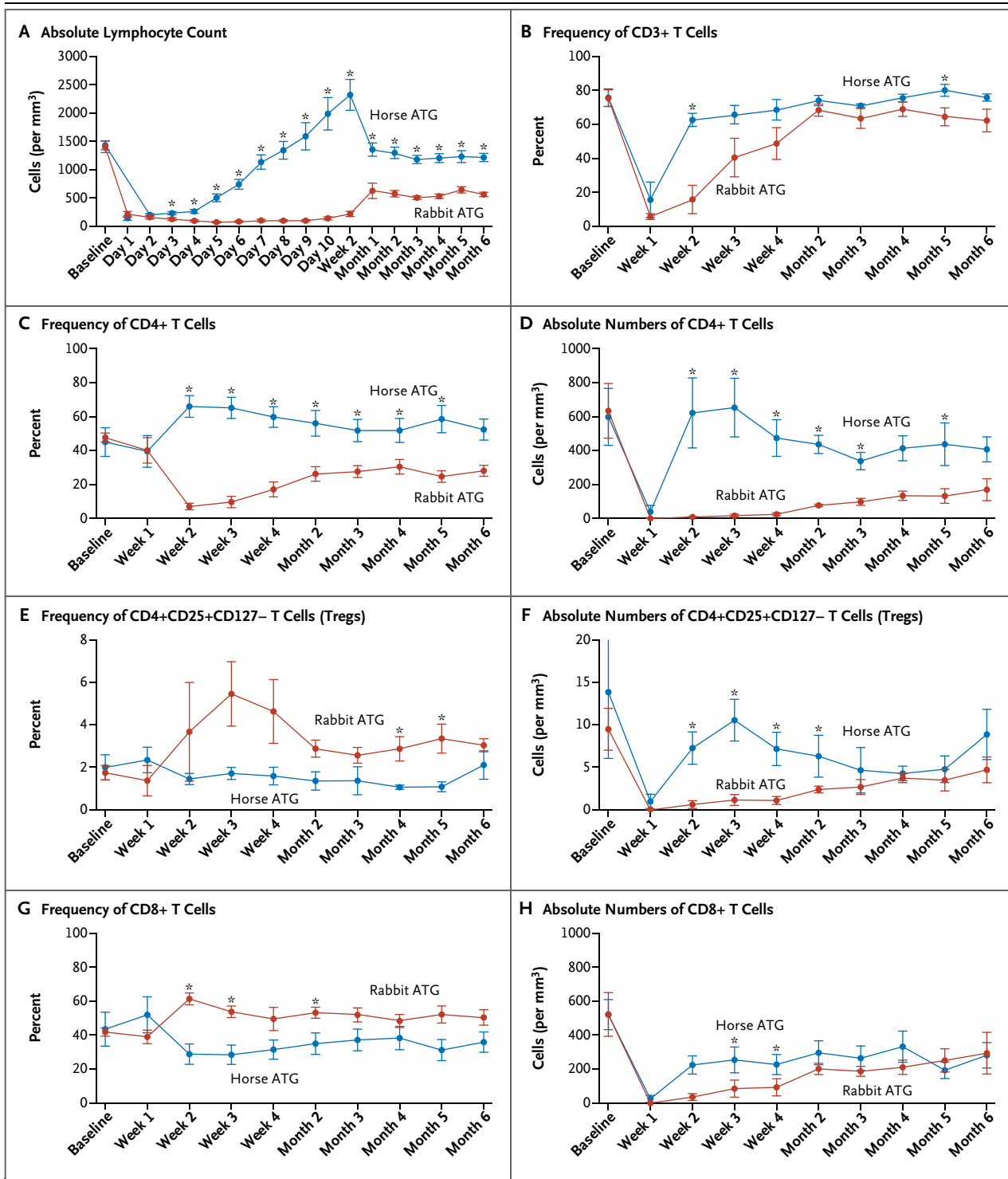
These results were unanticipated, given the success of rabbit ATG in treating relapsed and refractory severe aplastic anemia and the superiority of this agent in protecting kidney allografts.[13-16] The use of rabbit ATG as a first-line treatment for severe aplastic anemia was logical because it is more immunosuppressive than is horse ATG[17]; thus, a higher response rate and improved survival were anticipated. Our study was originally designed to test this hypothesis and was powered to detect a difference of 25 percentage points between the two groups. Because of the large clinical difference between the groups, the response and survival rates crossed statistical boundaries of significance at the conclusion of the study, with confidence intervals between groups that did not overlap for hematologic response and survival.

Our data raise questions about the mechanism by which hematopoiesis is restored after ATG administration in patients with severe aplastic anemia. Although the horse and rabbit preparations of ATG undergo apparently similar manufacturing processes, there are marked differences in vitro and in vivo between the two preparations. In human peripheral-blood mononuclear cells cocultured with different ATGs, an increase in the frequency of regulatory T cells was observed with rabbit ATG but not with horse ATG.[18] Furthermore, a marked difference in gene-expression profile was shown in human cells cultured with

A Data Censored for Stem-Cell Transplantation

P=0.04

No. at Risk

Horse ATG	60	39	23	10
Rabbit ATG	60	34	12	1

B Data Not Censored for Stem-Cell Transplantation

P=0.008

No. at Risk

Horse ATG	60	44	27	12
Rabbit ATG	60	41	22	6

Figure 2. Kaplan–Meier Curves of Overall Survival.

Panel A shows survival when data on patients were censored at the time of stem-cell transplantation; Panel B shows survival when stem-cell transplantation events were ignored.

either horse or rabbit ATG.[18] In humans, more prolonged lymphopenia follows rabbit ATG administration, and patterns of viral reactivation have been shown to differ between these two agents.[17]

Lot-to-lot variability among ATGs is unlikely to explain the large observed differences in outcomes. First, laboratory testing has not shown marked or consistent dissimilarity in cytotoxicity or antigen-binding specificities among multiple lots of horse and rabbit ATGs[24,25] nor among commercially available ATGs.[26] Second, in our clinical experience over a period of several decades, the

A **Absolute Lymphocyte Count**

B **Frequency of CD3+ T Cells**

C **Frequency of CD4+ T Cells**

D **Absolute Numbers of CD4+ T Cells**

E **Frequency of CD4+CD25+CD127− T Cells (Tregs)**

F **Absolute Numbers of CD4+CD25+CD127− T Cells (Tregs)**

G **Frequency of CD8+ T Cells**

H **Absolute Numbers of CD8+ T Cells**

response rates with horse ATG in sequential protocols among patients with previously untreated aplastic anemia have been nearly identical, at an average of 62% (including the current study).[10-12] Response rates with rabbit ATG among patients with refractory severe aplastic anemia have also appeared to be stable in separate studies conducted over a period of 10 years at our institution, at about 33%.[16,27] Third, because the preparation of rabbit ATG involves many animals, less variability would

Figure 3 (facing page). Mean (±SE) Depletion of Lymphocytes after ATG Administration.

The initial decrease in the absolute lymphocyte count was similar in the horse-ATG and rabbit-ATG groups, but lymphocyte counts remained lower longer after the use of rabbit ATG (Panel A). T cells (CD3+CD45+) decreased rapidly in both groups, with reconstitution in subsequent weeks (Panel B). There was a large difference in the kinetics of CD4+ T-cell depletion between the two groups, with a much lower frequency and lower absolute numbers after the use of rabbit ATG (Panels C and D). The frequency of regulatory T cells (Tregs; defined as CD4+CD25+CD127− for this analysis) was higher after the use of rabbit ATG, but absolute numbers were markedly lower because of more potent depletion of CD4+ T cells, as compared with depletion after the use of horse ATG (Panels E and F). The difference in depletion kinetics of CD8+ T cells was less striking between the two groups, as compared with the difference in CD4+ T cells (Panels G and H). Panel A shows data for all 120 patients. Panels B through H show data for 14 patients (7 from each group). Asterisks denote significant differences between the groups (P<0.05 on the basis of paired t-tests). For details on flow cytometric analysis, see the Methods section in the Supplementary Appendix.

be expected in this formulation. Fourth, the kinetics of lymphocyte depletion with either agent were consistent among the patients. Finally, secular trends in the response observed in the current study remained steady, and there were no significant differences in response rates among patients treated with different lots of ATG (data not shown).

Other, more plausible explanations probably account for our results. Horse and rabbit ATGs led to a similar depletion of CD8+ cytotoxic T cells, but there was a more profound depletion of CD4+ T cells after the use of rabbit ATG (Fig. 3). One possible inference is that the depletion of CD8+ T cells is linked to the success of treatment with ATG (horse or rabbit), as expected from the pathophysiology of aplastic anemia, but that the loss of CD4+ T cells after the use of rabbit ATG may

be detrimental. The CD4+ cell compartment is phenotypically and functionally heterogeneous. Contained within the large CD4+ cell population are regulatory T cells, which modulate immune responses. In the current study, the frequency of regulatory T cells was higher after the use of rabbit ATG than after the use of horse ATG (as predicted from tissue-culture experiments),[18,19] but this effect was negated by the more potent depletion of CD4+ T cells (Fig. 3E and 3F). CD4+ cells have other positive effects on hematopoiesis, and they may be important for hematologic recovery as well as for the promotion of tolerance in severe aplastic anemia (as after stem-cell transplantation).[28] In addition, horse serum might contribute to the recovery of hematopoiesis by stimulatory effects in the bone marrow.[29,30] More prolonged lymphopenia after the use of rabbit ATG might impair marrow recovery, because stimulatory cytokines derived from T cells are depleted.[31]

It is unclear whether further intensification of immunosuppression will yield superior outcomes among patients with severe aplastic anemia.[32] The addition of mycophenolate mofetil[10] or sirolimus[11] to horse ATG plus cyclosporine has not achieved this goal, and the use of more potent lymphocytotoxic agents (rabbit ATG and alemtuzumab[27]) in place of horse ATG has had inferior results. Although horse ATG is not available in many countries outside the United States, this agent combined with cyclosporine appears to be the most effective first-line immunosuppressive regimen for severe aplastic anemia.

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