ELSEVIER

Low-Dose Rabbit Antithymocyte Globulin Induction Therapy Results in Prolonged Selective Lymphocyte Depletion Irrespective of Maintenance Immunosuppression

O. Pankewycz, N. Leca, R. Kohli, P.K. Wallace, M. Said, L. Feng, M. Alnimri, S. Patel, and M.R. Laftavi

ABSTRACT

Rabbit antithymocyte globulin therapy (rATG) is a potent lymphocyte-depleting agent commonly used following renal transplantation to reduce the risk of acute rejection. Standard doses (7–10 mg/kg) of rATG result in profound lymphopenia and predispose patients to infection and malignancy. The effects of lower doses of rATG (LoD-rATG, 3–5 mg/kg) on peripheral blood lymphocytes (PBL) are as yet unknown. In this prospective clinical trial, PBL subsets were characterized by flow cytometry over 12 months following LoD-rATG therapy. All patients were initially treated with standard doses of tacrolimus, mycophenolic acid, and prednisone. At 3 months, patients were randomized to either lower doses of tacrolimus or sirolimus to examine the effects of maintenance immunosuppression on PBL reemergence. LoD-rATG therapy resulted in prolonged suppression of $CD19^+$ B cells, total $CD3^+$ T cells, as well as naïve and memory $CD4^+$ T cell and CD4/CD25/$Foxp3^+$ T-regulatory subsets irrespective of chronic immunosuppressive therapy. Selective depletion was only noted in the $CD4CD45RA^+$ naïve T-cell subset resulting in an altered memory/naïve $CD4^+$ ratio. LoD-rATG failed to deplete $CD8^+$ T cells, which increased their relative contribution to the total $CD3^+$ pool. All other lymphocyte subsets maintained near normal proportions. Thus, LoD-rATG therapy may lessen the adverse effects of full dose rATG while maintaining overall efficacy.

AS IMMUNOSUPPRESSIVE THERAPIES for renal transplantation progressed to include more potent and specific therapies, short-term graft and patient survival rates have continuously improved.[1,2] These results occurred despite the increasing use of nonstandard deceased donor organs, which are predisposed to early graft dysfunction and calcineurin toxicity.[1,2] To ameliorate these risks, it is becoming standard practice to include a lymphocyte-depleting agent at the time of transplantation. Currently, the vast majority of renal transplant recipients in the United States treated with induction therapy receive rabbit antithymocyte globulin (rATG; Thymoglobulin, Genzyme Transplant, Cambridge, Mass, USA).[3]

The immunologic consequences of rATG therapy are complex and ill defined; however, its most studied characteristic is rATG's effect on peripheral blood lymphocyte (PBL) depletion.[4,5] After renal transplantation, rATG-induced PBL reduction appears dose related.[4,6,7] Intensive therapy lasting 10 days with upward of 60 mg/kg total dose resulted in profound changes in PBL subsets lasting grater than 60 months.[6] More contemporary rATG dosing goals typically entail somewhat lower total amounts of 6 to 10 mg/kg, a dose that still leads to prolonged alterations in PBL phenotypes.[8,9] Such long-term effects of rATG may contribute to an increased risk for infection or malignancy without added immunologic efficacy in low-risk kidney transplant recipients.

From the Department of Surgery (O.P., M.S., L.F., M.A., S.P., M.R.), State University of New York, University at Buffalo, Buffalo General Hospital, Kaleidahealth, Buffalo, New York, USA; Department of Medicine (N.L.), University of Washington, Seattle, Washington, USA; Department of Medicine (R.K.), State University of New York, University at Buffalo, Buffalo General Hospital, Kaleidahealth, Buffalo, New York, USA; and Roswell Park Cancer Institute (P.K.W.), Buffalo, New York, USA.

This work was supported by an Investigator Initiated Research Grant, Genzyme Transplant Corp, Cambridge, Massachussetts, USA.

Address reprint requests to Oleh G. Pankewycz, Division of Transplantation, Buffalo General Hospital, 100 High Street, Buffalo, NY 14203. E-mail: opankewycz@kaleidahealth.org

0041-1345/11/$–see front matter
doi:10.1016/j.transproceed.2011.01.034

This current trial explores the effects of lower total dose of rATG induction therapy, 3 to 5 mg/kg, on PBL subsets over the course of 1 year after transplantation. In addition, in order to evaluate the effects of maintenance immunosuppression on PBL recovery, after 3 months patients either were randomized to either continued tacrolimus therapy or were switched to sirolimus.

PATIENTS AND METHODS

Patients

This randomized prospective clinical trial was conducted at Buffalo General Hospital, Kaleida Health Care System, with informed consent and approval from the Institutional Review Board of SUNY, University at Buffalo. Adult patients who received their first kidney transplant were eligible to participate. Exclusion criteria included a class I panel-reactive antibodies > 30%, simultaneous transplant of a second solid organ, history of malignancy, active pregnancy, total cholesterol > 300 mg/dL, triglycerides > 400 mg/dL, absolute neutrophil count <1000 cells/mm^3, and active viral infections with HIV or hepatitis B or C. Patients were recruited over a period from January 2008 until October 2010.

Study Design

All patients underwent implantation biopsy and received induction therapy with rATG (3–5 mg/kg total dose) given over 3 to 4 days along with 250 mg intravenous (IV) solumedrol. On day 2, patients were given 125 mg IV solumedrol and were started on tacrolimus (TAC) and sodium mycophenolate 720 mg twice a day. Oral prednisone was begun on day 3 at 30 mg/d and was tapered by 5 mg/wk until a maintenance dose of 5 mg/d was reached. For the first 3 months, TAC levels were kept between 10 and 15 ng/mL.

At 3 months, patients underwent surveillance transplant biopsy and flow cytometric analysis of PBL subsets. In the absence of subclinical rejection including borderline and higher grades, patients were randomized to two groups: low-dose TAC (LoTAC) with levels of 4 to 6 ng/mL or sirolimus (SRL) with levels of 5 to 10 (Abbott IMX Immunoassay). At 12 months, patients underwent a second surveillance biopsy and flow cytometric analysis.

Flow Cytometry

Phenotypic analysis of PBLs was performed as previously described using a six-color flow cytometer (FACSCanto II, BD Bioscience, San Jose, Calif, USA).[10,11] Lymphocyte subsets characterized included: $CD3^+$ (total T cells), $CD3^+CD4^+$, $CD3^+CD8^+$, $CD3^+CD4^+CD45RA^+$ (naïve), $CD3^+CD4^+CD45RO^+$ (memory), $CD19^+$ (B cells), $CD3^+CD4^+CD25^+Foxp3^+$ (regulatory T cells [Tregs]). Antibodies used were: CD3 PECy7 (SK7), CD4 PerCP (SK3), CD19 PECy7 (SJ25C1), CD25 APC (2A3), and CD45RA APC (HI100) were purchased from BD BioScience; CD45RO PE (UCHL.1) and CD127 PE (R34.34) were purchased from Beckman Coulter (Miami, Fla, USA); CD8 FITC (3B5) was purchased from Invitrogen (Carlsbad, Calif; USA); FOXP3 Ax488 (206D) and its isotype control (MOPC-21) were purchased from BioLegend (San Diego, Calif, USA). For comparison, flow results obtained in five normal, nontransplanted individuals were used as controls.

Statistics

Statistical analysis was performed by analysis of variance and Student *t* test for parametric data. Chi-square analysis was used for nonparametric data. Significance was considered to be $P < .05$.

RESULTS

A total of 58 patients were enrolled in the study. Six patients were noted to have subclinical inflammation on the 3-month protocol biopsy, five had borderline changes, and one had grade 1A rejection. These patients did not undergo randomization. Following randomization, one patient experienced acute rejection in the SRL arm. One patient in the LoTAC arm had subclinical borderline changes on the 12-month biopsy. The demographics for the remaining two groups of patients randomized to LoTAC or SRL are shown in Table 1. The two groups were well matched except for the presence of more expanded criteria donors in the LoTAC group.

The results of flow cytometry data obtained at 3 months for all 58 patients are shown in Table 2. Overall, the total number of $CD3^+$ lymphocytes was reduced by over 50%. A more dramatic reduction was noted for total, naïve, and memory $CD4^+$ subsets. $CD4^+$ naïve T cells appear especially vulnerable to low-dose rATG depletion since the proportion of $CD4^+RA^+$ cells to total $CD3^+$ cells fell from 29% to 12%. Although the absolute number of $CD4^+$ memory cells was also significantly reduced, their relative contribution to total $CD3^+$ numbers was unchanged (36% vs 32%, respectively). Given the difference in low-dose rATG effects on RA^+ and RO^+CD4^+ T cells, the ratio of memory to naïve $CD4^+$ cells increased from 1.2 to 2.6. Tregs also underwent a 75% reduction in absolute numbers yet the Treg to $CD4^+$ ratio remained unchanged at 0.04. The number of circulating $CD8^+$ T cells was not significantly reduced by low-dose rATG resulting in an increase in the relative proportion of $CD8^+$ cells within the entire $CD3^+$ set. Similar to $CD3^+$ T cells, $CD19^+$ B cells were equally affected by low-dose rATG.

The long-term effects of low-dose rATG comparing the influence of either LoTAC or SRL maintenance therapy on PBL reconstitution were evaluated by flow cytometry at 12 months. As shown in Table 3, there were no significant differences in the absolute numbers for any PBL subset between the LoTAC and SRL groups. Although total $CD3^+$ lymphocytes recovered to some extent in both groups, their absolute numbers remained substantially

Table 1. Patient Demographics

	LoTAC (n = 29)	SRL (n = 23)	Univariate P value
Age (y)	56.8 ± 12.4	51.1 ± 13.6	NS
Male (%)	72.4	65.2	NS
African-American (%)	13.8	13	NS
Living donor (%)	44.8	65.2	NS
Diabetes (%)	55.2	30.4	NS
Hypertension (%)	20.7	13.0	NS
Delayed graft function (%)	17.2	4.3	NS
Expanded criteria donors (%)	24.1	4.3	.05
Total rATG dose (mg/kg)	2.9 ± 0.79	3.0 ± 0.32	NS
HLA mismatch	3.7 ± 1.7	3.8 ± 1.4	NS

LoTAC, low-dose tacrolimus; SRL, sirolimus; rATG, rabbit antithymocyte globulin; HLA, human leukocyte antigen; NS, not significant.

Table 2. Absolute PBL Numbers at 3 Months (cells/mm³)

	Controls	LoD-rATG	% Reduction	*P* value
Total WBC	6454 ± 1749	6046 ± 2287		.69
$CD3^+$	1388 ± 301	557 ± 540	60	.001
$CD3^+CD4^+$	980 ± 207	269 ± 243	73	<.001
$CD3^+CD4^+CD45RA^+$	404 ± 216	63 ± 69	85	<.001
$CD3^+CD4^+CD45RO^+$	497 ± 175	170 ± 138	66	<.001
$CD3^+CD8^+$	334 ± 140	226 ± 244	33	.33
$CD19^+$	238 ± 125	119 ± 95	50	.01
$CD4^+CD25^+Foxp3^+$	48 ± 13.9	10 ± 8.6	80	<.001

Results are mean ± standard deviation. PBL, peripheral blood lymphocytes; LoD-rATG, low-dose rabbit antithymocyte globulin; WBC, white blood cells.

lower than in controls. In both groups, total $CD4^+$ T cells as well as both their memory and naïve subset numbers remained equally depressed. Memory $CD4^+$ cells continued a slow recovery process irrespective of maintenance immunotherapy, while naïve cells remained essentially unchanged. This differential recovery of $CD4^+$ cell subsets resulted in a still greater memory to naïve cell ratio of 4.2. Tregs also slowly recovered but their absolute numbers in both the LoTAC and SRL groups were equally depressed, remaining approximately half of control values. The ratio of Tregs to other $CD4^+$ cell populations remained constant except for the ratio of Tregs to naïve $CD4^+$ T cells, which was substantially increased from approximately 12% to 36%.

At 1 year, the absolute number of $CD8^+$ PBLs in both maintenance immunosuppressive groups remained within the normal range. Thus, $CD8^+$ PBLs continue to comprise a greater percentage of total $CD3^+$ cells. Over the course of 1 year, the number of circulating $CD19^+$ B lymphocytes showed no tendency to increase. Although not significant, the number of B cells in the LoTAC group was slightly greater than in the SRL patients ($P = .09$).

DISCUSSION

Induction immunosuppressive therapy for renal transplantation with rATG has proven short- and long-term benefits.[12,13] The potential deleterious effects of rATG in relation to the risks of infection and malignancy, in addition to the significant associated costs, demand the exploration of the efficacy of this drug at lower exposures. The duration of therapy and the total amounts of rATG required to achieve protection from rejection and graft failure remain uncertain. A variety of successful therapeutic strategies have been employed including both short and long courses consisting of total doses ranging from 3 to 10.5 mg/kg.[7,12–14] To a great extent, the dose requirement for rATG may depend on the character of the patients to be treated. We now demonstrate that low-dose rATG (3 mg/kg) is highly effective in preventing clinically evident as well as subclinical acute rejection when given as induction therapy to a low-risk recipient population.

In this trial, changes in PBL subset phenotypes were measured as a surrogate marker of low-dose rATGs in vivo efficacy. Similar to higher doses of rATG, low-dose rATG therapy led to severe and long-term depletion of the total $CD4^+$ population that was especially notable in the naïve $CD4^+$ subset.[6–8] The resultant increase in the $CD4^+$ memory/naïve cell ratio was primarily caused by selective naïve $CD4^+$ cell depletion. The modest long-term reduction in B-cell numbers seen with low-dose rATG also mirrored results observed with higher total doses.[6,9,15] The $CD8^+$ T-cell population appears resistant to rATG-depleting effects irrespective of dose.[6,7] Thus, in terms of the aforementioned PBL subsets, low-dose rATG therapy led to similar long-term changes in PBL subsets as was previously reported with higher doses.

Low-dose rATG therapy also had profound and lasting depleting effects on Tregs. Moreover, the Treg/$CD4^+$ cell ratios were not altered at 3 and 12 months irrespective of maintenance immunosuppression. These results are in contrast to the Treg-promoting effect observed in transplant recipients treated with higher doses of rATG.[8,9] This discrepancy may be due to a dose effect. In vitro, rATG was shown to induce Tregs at concentrations of 5 to 100 μcg/mL, which are normally achieved in vivo after 7 to 11 days of therapy at daily doses of 1.25 to 1.5 mg/kg.[16–18] Thus, low-dose rATG therapy may not have achieved the concentrations required induce Tregs. Since a greater Treg/$CD4^+$ ratio in PBLs correlates with the better graft outcomes, the use of higher doses of rATG in high-risk patients may prove to be more efficacious.[19] The effectiveness of low-dose rATG induction therapy in high-risk patients remains to be studied.

Recent studies suggest that SRL maintenance immunosuppression promotes the generation of Tregs following rATG induction therapy.[8] Compared to TAC, we failed to observe a differential effect of SRL on Treg numbers or percentages. This may have been due to the use of TAC in all patients for the first 3 months, negating any beneficial SRL effect. Alternatively, Treg numbers may increase in the SRL group given a longer period of observation.

A criticism of this study may be the use of normal controls as a comparator with our transplant patients. However, the numbers of PBLs and their subsets in our

Table 3. Absolute PBL Numbers at 12 Months (cells/mm³)

	LoTAC ($n = 18$)	SRL ($n = 12$)
Total WBC	8050 ± 2690	6842 ± 2331
$CD3^+$	657 ± 265*	712 ± 442*
$CD3^+CD4^+$	348 ± 167*	347 ± 165*
$CD3^+CD4^+CD45RA^+$	61 ± 52*	62 ± 98*
$CD3^+CD4^+CD45RO^+$	254 ± 150*	263 ± 108*
$CD3^+CD8^+$	245 ± 148†	311 ± 327†
$CD19^+$	132 ± 89‡	85 ± 32*
$CD4^+CD25^+Foxp3^+$	19 ± 36†	25 ± 15‡

Results are mean ± standard deviation.
PBL, peripheral blood lymphocytes; LoTAC, low-dose tacrolimus; SRL, sirolimus; WBC, white blood cells.
*$P < .001$ versus control.
†P = not significant versus control.
‡$P < .05$ versus control.

controls are well within the normal ranges and closely mirror those obtained in similar studies of patients prior to transplantation.[6–8] In summary, we demonstrate that low-dose rATG is an effective induction strategy that protects from clinical and subclinical rejection when used in low-risk patients. The short- and long-term changes in PBL subsets induced by low-dose rATG closely mimic those obtained with higher doses except for the Treg population. Given the current protocol, we were not able to discern a differential effect of maintenance immunosuppression on PBL subset numbers or proportions. The in vivo consequences of these differences remain to be studied.

REFERENCES

1. US Renal Data System, USRDS 2010 Annual Data Report: Atlas of Chronic Kidney Disease and End-Stage Renal Disease in the United States. Bethesda, MD: National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases; 2010

2. 2009 Annual Report of the U.S. Organ Procurement and Transplantation Network and the Scientific Registry of Transplant Recipients: Transplant Data 1999–2008., Rockville, MD: U.S. Department of Health and Human Services, Health Resources and Services Administration, Healthcare Systems Bureau, Division of Transplantation

3. Gaber AO, Knight RJ, Patel S, et al: A review of the evidence for use of thymoglobulin induction in renal transplantation. Transplant Proc 42:1395, 2010

4. Merion RM: 2008 SRTR report on the state of transplantation. Am J Transplant 9:867, 2008

5. Mueller TF: Mechanisms of action of thymoglobulin. Transplantation 84(11S):S5, 2007

6. Muller TF, Grebe SO, Neumann M, et al: Persistent long-term changes in lymphocyte subsets induced by polyclonal antibodies. Transplantation 64:1432, 1997

7. Wong W, Agrawal N, Pascual M, et al: Comparison of two doses of thymoglobulin used as a short course for induction in kidney transplantation. Transpl Int 19:629, 2006

8. Gurkan S, Luan Y, Dhillon N, et al: Immune reconstitution following rabbit antithymocyte globulin. Am J Transplant 10:2132, 2010

9. Morelon E, Lefrancois N, Besson C, et al: Preferential increase in memory and regulatory subsets during T-lymphocyte immune reconstitution after Thymoglobulin induction therapy with maintenance sirolimus vs cyclosporine. Transpl Immunol 23:53, 2010

10. Grant J, Bourcier K, Wallace S, et al: Validated protocol for Foxp3 reveals increased expression in type 1 diabetes patients. Cytometry B Clin Cytom 76B:67, 2009

11. Stewart CC: Multiparameter analysis of leukocytes by flow cytometry. Methods Cell Biol 33:427, 1990

12. Brennan DC, Flavin K, Lowell JA, et al: A randomized, double-blinded, comparison of Thymoglobulin versus Atgam for induction immunosuppressive therapy in adult renal transplant recipients. Transplantation 67:1011, 1999

13. Hardinger KL, Rhee S, Buchanan P, et al: A randomized, double-blinded, comparison of Thymoglobulin versus Atgam for induction immunosuppressive therapy: 10-year results. Transplantation 86:947, 2008

14. Agha IA, Rueda J, Alvarez A, et al: Short course induction immunosuppression with thymoglobulin for renal transplant recipients. Transplantation 73:473, 2002

15. Zand M: Therapeutic antibody agents for B-cell immunomodulation in renal transplantation. Transplantation 84:S11, 2007

16. LaCorcia G, Swistak M, Lawendowski C, et al: Polyclonal rabbit antithymocyte globulin exhibits consistent immunosuppressive capabilities beyond cell depletion. Transplantation 87:966, 2009

17. Sewgobind VDKD, Van der Laan LJW, Kho MML, et al: Characterization of rabbit antithymocyte globulins-induced $CD25^+$ regulatory T cells from cells of patients with end-stage renal disease. Transplantation 89:655, 2010

18. Lopez M, Clarkson MR, Albin M, et al: A novel mechanism of action for anti-thymocyte globulin: induction of $CD4^+CD25^+Foxp3^+$ regulatory T cells. J Am Soc Nephrol 17:2844, 2006

19. Bas J, Mestre M, Grinyo JM, et al: Peripheral blood lymphoid subsets and long-term clinical course of kidney recipients: a longitudinal study. Cytometry 34:103, 1998