In Vitro Influence of Polyclonal Anti-Thymocyte Globulins on Leukocyte Expression of Adhesion Molecules

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Objectives: **Polyclonal anti-thymocyte globulins (ATGs) are drugs used in the induction of immunosuppression, in the treatment of acute rejection, and in the therapy of hematologic disorders. Treatment with ATGs can produce adverse effects due to cross-reacting antibodies directed against nonmyeloid cells. This study sought to evaluate the interaction of ATGs and some adhesion molecules expressed on the surface of neutrophils and lymphocytes.**

Materials and Methods: **We determined the effects of different doses of 3 polyclonal ATGs on the activation and expression of lymphocyte and neutrophil adhesion molecules in whole blood by means of flow cytometry.**

Results: **ATG treatment reduced the percentage of lymphocytes gated for CD18 and CD62L, as well as the expression of CD11b, CD18, and CD62L in a dose-dependent manner. ATGs modulated the percentage of gated neutrophils for CD18. Although ATG treatment did not affect CD11b or CD62L gating in neutrophils, it did regulate expression of these adhesion markers.**

Conclusions: **Our results show that ATGs can modify the expression levels of some of the main leukocyte adhesion molecules that are responsible for the characteristic cellular adhesion after ischemia/reperfusion. These properties of ATGs may contribute to reduced leukocyte infiltration after solid-organ transplantation.**

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Polyclonal anti-thymocyte globulins (ATGs) are a group of immunosuppressive drugs currently used to prevent and treat acute rejection after organ transplantation (including steroid-resistant rejection) [1], as conditioning regimens for bone marrow transplantation, and to treat hematologic disorders such as aplastic anemia or graft-versus-host disease [2]. ATGs have a wide range of effects including depletion and induction of apoptosis of peripheral T lymphocytes. To a lesser extent, depletion of T lymphocytes is also found in the spleen and lymph nodes [3]. ATGs also are able to modulate B lymphocytes [4] as well as the expression of various leukocyte adhesion molecules, including L-selectin [5]. These properties of ATGs may modify the characteristic cellular adhesion following ischemia/reperfusion. Differences in the production of commercially available ATGs (both rabbit- and equine-derived) have led to differences in the dosages administered and the effects observed in clinical practice [6]. Essentially, the immunization of rabbits with human thymocytes or cultured cell lines is unlikely to be specific for a single human cell type [7]. Because thymocytes express antigens common to many human cell types, polyclonal ATGs could potentially recognize other circulating antigens, making the modulation of adhesion molecules upon neutrophils and monocytes possible. Standardization of production as well as development of quality control procedures has improved the consistency of ATGs, providing a satisfactory clinical tolerance and an increased specificity for T lymphocytes. However, phenomena such as dose-dependent neutropenia or thrombocytopenia are still occasionally reported, indicating the presence of antibodies directed against nonmyeloid cells in these preparations (7). Those antibodies could limit the adhesion properties of nonmyeloid cells in cases of ischemia/reperfusion

injury or delayed graft function. The aim of our study was to investigate the effects of 3 different ATGs on neutrophil and leukocyte adhesion molecules (CD11b, CD18, and CD62L) by means of flow cytometry.

Materials and Methods

Reagents

Three different ATG preparations were used in this study: ATG-Fresenius-S (ATG-1; batch 010 H-2, Fresenius Biotech, Munich, Germany), Thymoglobulin (ATG-2; Ch-B.: TH013-7, IMTIX-SangStat, Lyon, France), and Tecelac (ATG-3; Batch 5-190012, Biotest AG, Dreieich, Germany). Human thymocytes were used as immunogens to produce both ATG-2 and ATG-3, whereas a cultured line of Jurkat T cells was used to generate ATG-1. These preparations were ready-to-use liquid (H_2O) solutions containing 5.5 mg/mL (ATG-2) and 20 mg/mL (ATG-1 and ATG-3) of purified rabbit immunoglobulin IgG. A lymphocytotoxicity test, according to the method of Terasaki [8], was performed to determine the lowest concentration of ATG at which more than 50% of the lymphocytes were depleted after a 10-minute incubation with a complement. This concentration was noted as a titer (ATG-1, 1:50; ATG-2, 1:200; and ATG-3, 1:1600). A 5-fold concentration of these titers was employed as a cytotoxic dosage (ATG-1/x5; ATG-2/x5; and ATG-3/x5). Phosphate buffered saline (PBS) (without calcium/magnesium; 0.18%), Trypan blue (0.4%), and sodium citrate (3.1%) were obtained from the pharmacy office of the hospital.

Antibodies

The fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies against CD11b and CD18, and the R-phycoerythrin (R-PE)-conjugated antibody against CD62L, were purchased from BD Biosciences (San José, Calif, USA). CD11b modulates the adherence of polymorphonuclear neutrophils and monocytes to fibrinogen and positive ICAM-1 endothelia. CD18 is directed against the beta chain of the lymphocyte function-associated antigen-1. CD18 is expressed on mature immunocompetent lymphocytes and their neoplastic counterparts, granulocytes, and monocytes, and is involved with cell/cell and cell/extracellular adhesion in immune and inflammatory responses. CD62L is a vascular adhesion molecule constitutively expressed on granulocytes, monocytes, and a vast array of circulating lymphocytes. CD62L (L-selectin) is important for lymphocyte homing and adhesion to endothelial cells of postcapillary venules of peripheral lymph nodes, especially during the early phases of the adhesion cascade.

Blood collection

The protocol conformed with the ethical guidelines of the 1975 Helsinki Declaration. After informed consent had been obtained, whole blood (15 mL) was collected by venipuncture with minimal stasis from 12 healthy volunteers (6 men and 6 women). Blood was drawn into plastic syringes, and the first 5 mL was discarded to prevent contamination. The blood was anticoagulated with 3.1% sodium citrate and distributed into sterile 14-mL polypropylene tubes (Greiner Bio-One, Solingen-Wald, Germany).

Preparation for flow-cytometry analyses

ATG 1, 2, or 3 (0.1 mL) diluted in PBS was added to 0.9 mL blood and incubated for 10 minutes at room temperature. Samples (5 μL) were then mixed with 100 μL PBS in polystyrene round-bottom tubes (5 mL Falcon, BD-Pharmingen, Heidelberg, Germany) and incubated with 5 μL undiluted FITC- and PE-conjugated antibodies for 10 minutes at room temperature in the dark. The reaction was stopped with 900 μL of PBS.

Flow cytometry analyses

Flow cytometry was performed with a FACSort flow cytometer (BD Biosciences) equipped with a 15-mW argon laser emitting at 488 nm. The fluorescence signals of FITC and PE were detected with 530/30 nm (channel 1) and 585/42 nm band pass filters (channel 2) with correction of the spectral overlap by color compensation. Calibration of the flow cytometer was performed using standard fluorescent microbeads (CaliBRITE, BD Biosciences). Analysis of the fluorescence properties of 50,000 events was performed using CellQuest software (BD Biosciences). Both the intensity of labeling and the gating of electronic windows for lymphocytes and neutrophils were analyzed. The results are expressed as the percentage of gated cells for each particular antibody, defining the percentage of cells expressing the adhesion molecule studied, and the mean fluorescence intensity (MFI), which explains the intensity of expression of the analyzed molecule on the cell surface.

Group design and statistical analyses

Samples were distributed according to the ATG and the dosage employed, and 6 groups were generated, 3 of them with a standard dosage (ATG1, -2, -3) and 3 of them with a toxic dosage (ATG1, -2 ,-3 /x5) (n = 6/group). A control group was included, in which whole blood was incubated with PBS instead of ATG (n = 6). Results are expressed as means ± standard deviation. Statistical analyses were performed with a one-way ANOVA. A posteriori comparisons between the ATG-treated and control groups were performed using the Dunnett test. Post hoc comparisons within the ATG-treated groups were done with the Tukey test. A *P* value less than .05 was accepted as statistically significant.

Results

Effect of polyclonal ATGs upon expression of leukocyte adhesion molecules

The percentage of gated lymphocytes for CD11b after incubation with ATGs ranged between 20%-30%, but this difference was not statistically significant (Figure 1A). The mean MFI for this antibody was significantly reduced in the ATG-1/x5 and ATG-3/x5 groups (Figure 1B). The percentage of gated lymphocytes for CD18 was significantly higher in the control group in comparison with the ATG groups (Figure 1A). Although the MFI for CD18 was lower in the ATG groups, no statistically significant differences between the groups were found (Figure 1B). ATG treatment significantly reduced the percentage of gated lymphocytes for CD62L in the high-dose ATG groups, while there was no significant difference between low-dose ATG groups and controls ($P < .05$) (Figure 1A). MFI for L-selectin was significantly reduced in all the ATG groups, with the exception of ATG-2 ($P < .05$) (Figure 1B).

ATG treatment did not affect the percentages of gated neutrophils for CD62L and CD11b but did significantly lower the percentage for CD18 (Figure 2A). ATG-1/x5 significantly increased MFI for CD11b, whereas ATG treatment did not change the MFI for CD18 (Figure 2B). According to the MFI, no differences between the ATG groups and controls were found for CD18, although ATG-1/x5 demonstrated significantly higher values for CD11b. All ATG treatments (except the ATG-2 and ATG-3 groups) significantly reduced the MFI for CD62L (Figure 2B).

Differences between the 3 ATGs in standard and 5-fold dosages

Increased doses of ATG did not significantly change expression of CD11b on lymphocytes at both dosages studied (Figure 1A). However, there was a significant reduction of MFI in the ATG-1/x5 and ATG-3/x5 groups compared with the low-dose ATG groups (Figure 1B). All high-dose groups showed lower percentages of gated lymphocytes for CD18, although the MFI for this molecule was not affected ($P < .05$). Furthermore, there were differences within the standard-dose ATG groups, with ATG-1 and ATG-3 demonstrating significantly lower percentages of gated lymphocytes for CD18 than for the ATG-2 group

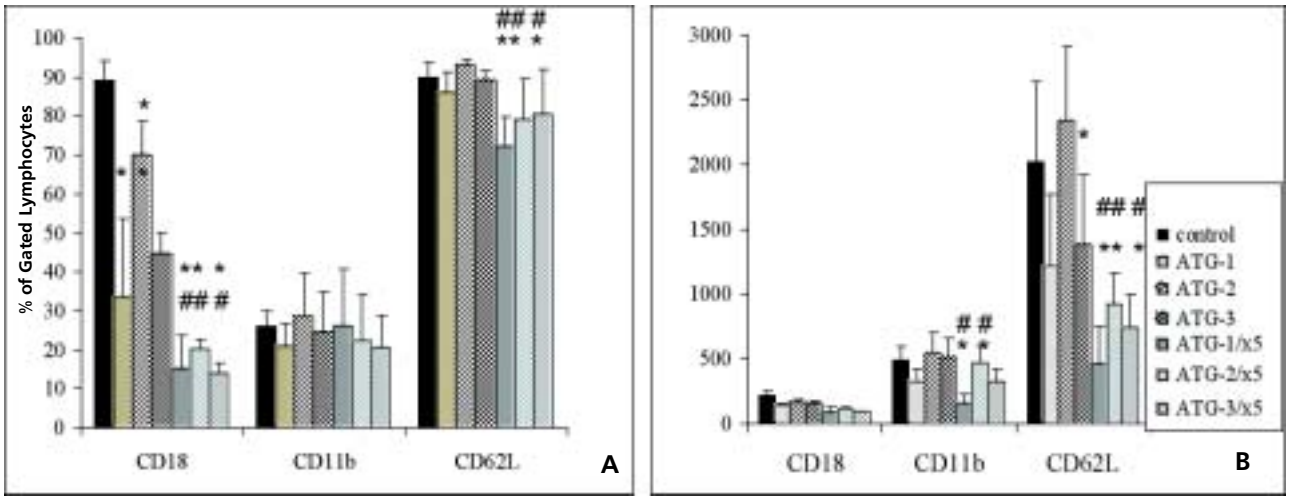

Figure 1. Comparison of the percentage of gated lymphocytes (**A**) and mean intensity of fluorescence (**B**) between the different dosages of ATG and control groups. *ATG vs control; #ATG vs ATG/x5. $P < .05$.

(Figures 1A and B). For CD62L, the 5-fold dosage ATG groups demonstrated lower values than did the low-dose ATG groups in terms of both the percentage of gated lymphocytes and MFI. The ATG-1 and ATG-3 groups showed a reduced MFI compared with ATG-2 in the standard-dose groups, and ATG-1/x5 showed significantly lower values for CD62L than did ATG-2/x5 and ATG-3/x5 ($P < .05$) (Figures 1A and 1B).

There were no differences within the ATG groups regarding percentages of gated neutrophils for CD11b (Figure 2A). ATG-1 and ATG-3 in the 5-fold dosage showed higher values of MFI than did the other ATG groups (Figure 2B). Furthermore, the ATG-1/x5 group showed a significant increase of MFI when compared with the standard-dose groups (Figure 2B). The CD18 percentage of gated neutrophils within the ATG groups was significantly higher in the ATG-1/x5, ATG-2, and ATG-3 standard-dosage groups compared with the ATG-1, ATG-2/x5, and ATG-3/x5 groups ($P < .05$) (Figure 2A). However, the ATG-2 group demonstrated higher values than all the other groups. There were no differences in the percentage of gated neutrophils for CD62L within the ATG groups. However, all ATGs showed significantly lower intragroup MFI levels in the 5-fold dosage (Figure 2B). Furthermore, ATG-1 and ATG1/x5 showed lower intensity of expression than did the other ATG groups at both dosages.

Discussion

Polyclonal ATGs are a group of commonly used immunosuppressive agents used in transplantation and hematology. ATGs deplete peripheral blood T cells through active cell death by both complement-dependent lysis and activation-associated apoptosis [9]. However, ATGs are not specific for T lymphocytes; they contain antibodies that bind to other circulating cells including granulocytes, B lymphocytes, and thrombocytes [7]. Preville and coworkers have shown that nonhaemadsorbed ATGs produce antibodies directed against circulating erythrocytes, neutrophils, and platelets [6]. We have previously demonstrated that ATGs can influence the expression of adhesion molecules on unstimulated thrombocytes [10], and we hypothesized that ATGs containing antibodies against leukocyte adhesion molecules could reduce tissue infiltration and inflammation in cases of ischemia/reperfusion injury [11].

The data presented in this study show that polyclonal ATGs modulate the in vitro expression of leukocyte adhesion molecules. ATG treatment did not change the percentage of gated lymphocytes for CD11b, but did dose-dependently decrease MFI. Because this β2 integrin plays a crucial role in the firm attachment of leukocytes to the endothelium during the inflammatory response, down-modulation of CD11b may reduce lymphocyte infiltration after reperfusion. CD11b expression on neutrophils was not modulated by exposure to ATGs, which is consistent with the data presented by Michallet and coworkers [5]. Since the CD11b antigen is present on approximately 30% of peripheral blood lymphocytes (including most natural killer lymphocytes, mature neutrophils, and monocytes [12]), down-modulation

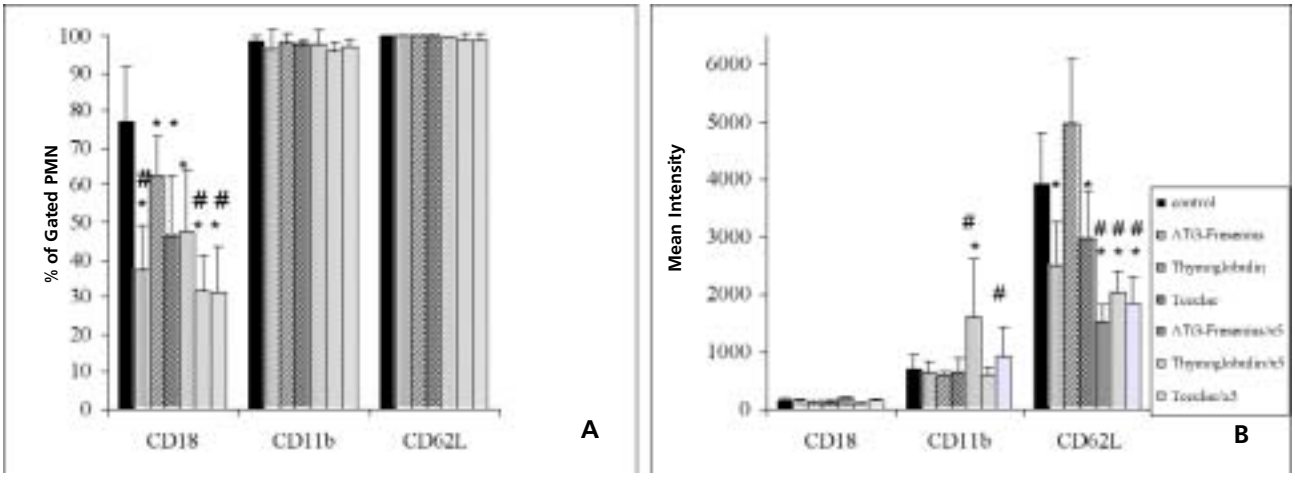

Figure 2. Comparison of the percentage of gated polymorphonuclear leukocytes (PMN) (**A**) and mean intensity of fluorescence (**B**) between the different dosages of ATG and control groups. *ATG vs control; # ATG vs ATG/x5. $P < .05$.

of its adhesion properties might reduce leukocyte-endothelium interactions and thus limit the role of these cells in cases of ischemia/reperfusion injury and delayed graft function.

ATG treatment dose-dependently reduced the percentage of gated lymphocytes for CD18. The minimal changes in MFI even in the control group are possibly related to the lack of lymphocyte activation in our study. CD18 analysis in neutrophils showed similar results. Although the intensity of expression remained low both in the control and in the ATG groups, CD18 levels were regulated differently by ATGs from different sources. Although ATGs from thymocytes (ATG-2 and ATG-3) induced a decrease in the percentage of CD18-positive neutrophils in a dose-dependent manner, ATG-1 increased CD18-positive neutrophils in the 5x group. ATG-1 is derived from a Jurkat T-cell line, being possible that it contains fewer antibodies against nonmyeloid cells than thymocyte-derived polyclonal antibodies. In fact, ATGs immunized against human thymocytes lead to a broad T-cell depletion, and ATGs immunized against the Jurkat T-cell line lead mainly to depletion of activated T cells (13).

Our data, in contrast with those of Michallet and coworkers [5], also suggest a dose-dependent specific reduction of the CD62L-gated lymphocytes and MFI after increased doses of ATGs, although no significant differences between the control and standard-dose ATG groups were found. This may explain the decrease in leukocyte adhesion after administration of ATGs [11]. CD62L percentages of gated neutrophils were not affected by incubation with ATGs either in standard or in high dosages. However, the intensity of expression followed the same pattern as lymphocytes, showing a dose-dependent decrease of expression after ATGs.

In conclusion, ATGs can regulate the expression of adhesion molecules (CD11b, CD18, and CD62L) on lymphocytes and neutrophils. These properties of ATGs may be helpful in reducing leukocyte-endothelial interactions, adherence, and posterior infiltration characteristic of ischemia/reperfusion injury or rejection episodes after organ transplantation.

References

1. Cosimi AB. Antilymphocyte globulins and monoclonal antibodies. In: Morris PJ, ed. Kidney Transplantation: Principles and Practice. Philadelphia, Saunders; 1988, p 343
2. Tichelli A, Gratwohl A, Nissen C, Signer E, Stebler-Gysi C, Speck B. Morphology in patients with severe aplastic anemia treated with antilymphocyte globulin. Blood 1992; 80: 337-345
3. Preville X, Flacher M, LeMauff B, Beauchard S, Davelu P, Tiollier J, et al. Mechanisms involved in antithymocyte globulin immunosuppressive activity in a nonhuman primate model. Transplantation 2001; 71: 460-468
4. Rebellato LM, Gross U, Verbanac KM, Thomas JM. A comprehensive definition of the major antibody specificities in polyclonal rabbit antithymocyte globulin. Transplantation 1994; 57: 685-694
5. Michallet MC, Preville X, Flacher M, Fournel S, Genestier L, Revillard JP. Functional antibodies to leukocyte adhesion molecules in antithymocyte globulins. Transplantation 2003; 75: 657-662
6. Preville X, Nicolas L, Flacher M, Revillard J. A quantitative flow cytometry assay for the preclinical testing and pharmacological monitoring of rabbit antilymphocyte globulins (rATG). J Immunol Methods 2000; 245: 45-54
7. Greco B, Bielory L, Stephany D, Hsu SM, Gascon P, Nienhuis A, et al. Antithymocyte globulin reacts with many normal human cell types. Blood 1983; 62: 1047-1054
8. Terasaki PI, McClelland J. Microdroplet assay of human serum cytotoxins. Nature 1964; 204: 998-1000
9. Genestier L, Fournel S, Flacher M, Assossou O, Revillard JP, Bonnefoy-Berard N. Induction of Fas (Apo-1, CD95)-mediated apoptosis of activated lymphocytes by polyclonal antithymocyte globulins. Blood 1998; 91: 2360-2368
10. Beiras-Fernandez A, Walther S, Muenzing S, Thein E, Hammer C. In vitro assessment of dose-dependent platelet activation by polyclonal antithymocyte globulins: a flow-cytometric analysis. Transplantation 2004; 78: 751-754
11. Beiras-Fernandez A, Thein E, Chappell D, Hammer C. Morphological assessment of reperfusion injury in a concordant xenogeneic primate model. Ann Transplant 2003; 8(3): 50-52
12. Landay A, Gartland L, Clement LT. Characterization of a phenotypically distinct subpopulation of Leu-2+ cells that suppresses T cell proliferative responses. J Immunol 1983; 131: 2757-2761
13. Kroger N. Rabbit-IgG levels in patients receiving thymoglobulin as part of conditioning before unrelated donor stem cell transplantation. Haematologica 2005; 90:869B